“Identification of the role of *Arabidopsis* ATAF-type NAC transcription factors in plant stress and development”

Dissertation

for the award of the degree

“Doctor of Philosophy” (Ph.D.)

Division of Mathematics and Natural Sciences

of the Georg-August-Universität Göttingen

within the doctoral program Biology

of the Georg-August University School of Science (GAUSS)

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Göttingen 2014

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Date of oral examination: 16th April 2014

Dedicated to my loving ***Family***

Contents

1. Introduction

Plants being sessile in nature do not enjoy the freedom of movement to escape harsh environmental conditions or pathogen invasions. In order to survive they have evolved efficient means of responding to different stresses that can be encountered. Physical barriers in the form of cell wall, cuticle and surface structures such as thorns, trichomes etc. as well as pre-formed anti-microbial chemicals function as a first layer of unspecific defense response against all kinds of threats. A second layer of induced stress response is activated when the first barrier is insufficient to contain the threat. This induced response involves recognition of pathogens or harmful substances and a response via production of counteractive chemicals/enzymes or even cell death. There have been extensive studies aiming to understand specific defense and stress responses. However, the complexities arising from the interconnection of many stress-induced pathways leaves much to be unraveled.

1.1 Xenobiotic stress response in plants

Plants often have to deal with exogenous xenobiotic compounds (e.g. chemical pesticides or toxins secreted by microorganisms and other plants) or endogenously produced toxic molecules. Detoxification and disposal of toxic compounds of both origins is essential for plant survival and is carried out via three main steps (Figure 1.1). The first phase called transformation involves enzymes that oxidize, reduce or hydrolyze the toxic substance thus making them accessible for the next phase. The second phase is conjugation wherein the transformed substrate is conjugated to moieties like glutathione or glucose making them less or non-toxic. Numerous plant species are known that can exhibit tolerance to toxic benzoxazinoids by rapidly metabolizing them to less phytotoxic glucoside derivatives (Baerson et al., 2005). The third and final phase involves compartmentalization of the conjugates into cell vacuoles or deposition into the apoplast (Coleman et al., 1997; Dixon et al., 1998; Li et al., 2002; Sandermann Jr., 1992). For example, it was shown recently that the xenobiotic monochlorobimane is conjugated to glutathione in the cytosol and then

transported to the vacuole where *gamma-glutamyl transpeptidase 4* (*GGT4*) initiates its further degradation (Grzam et al., 2007).

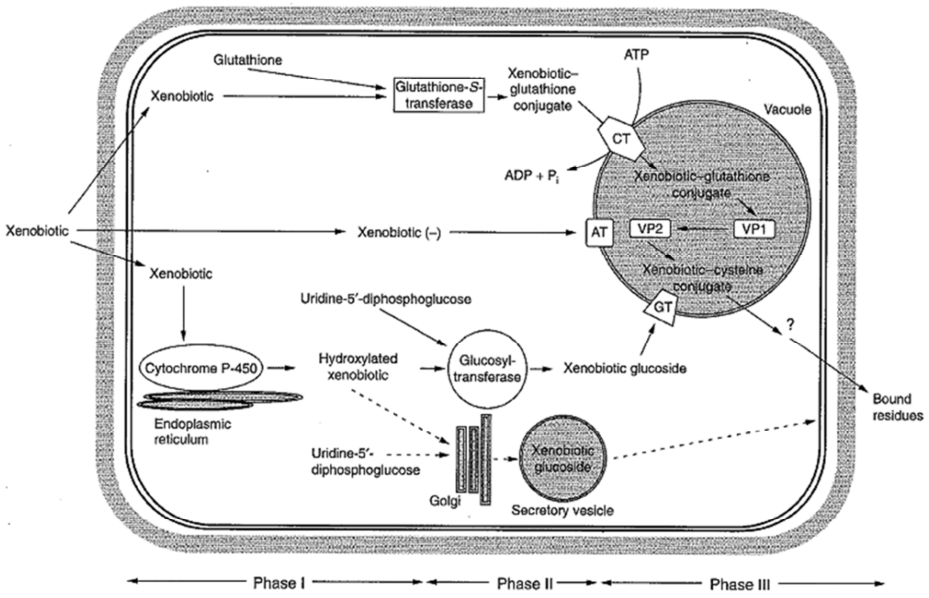


Figure 1.1 Detoxification of xenobiotics in plants (Coleman et al., 1997)

The enzyme-catalyzed reactions responsible for the detoxification of xenobiotics in plants are either localized in the cytosol or associated with the endomembrane system. The broken arrows represent a proposed pathway for glucosylation of xenobiotics in the Golgi compartment, followed by transport of metabolites into the vacuole or further release into the apoplast via exocytosis. Abbreviations: CT, glutathione conjugate transporter; AT, ATP-dependent anion transporter; GT, ATP-dependent glucoside-conjugate transporter; VP, vacuolar peptidase.

TGA factors, belonging to the subfamily of basic domain/leucine zipper (bZIP) transcription factors (TFs), are required for the induction of several genes related to response against xenobiotic compounds [e.g. *NITRILASE 4* (*NIT4*), *ALDO KETO REDUCTASE FAMILY 4 MEMBER C9* (*AKR4C9*)] (Klinedinst et al., 2000; Mueller et al., 2008). Moreover, it was shown that safeners like isoxadifen-ethyl and mefenpyr-

diethyl induce the xenobiotic detoxification response when applied to *Arabidopsis*; the majority of these safener-responsive genes depended on class II TGA TFs (*TGA2*, *TGA5* and *TGA6*) and/or SA for induction (Behringer et al., 2011). TGA factors bind *activation sequence-1* (*as-1*) elements which are overrepresented in promoters responsive to xenobiotic stress (Baerson et al., 2005; Mueller et al., 2008). The role of class II TGA TFs in detoxification was also emphasized when they were shown to interact with the *Arabidopsis* GRAS family protein *SCARECROW-LIKE 14 (SCL14)*. SCL14 is recruited to target promoters by the TGA factors and functions as a transcriptional co-activator (Fode et al., 2008). The TGA/SCL14 complex is important for the activation of several genes that are induced by xenobiotic stress. Application of toxic chemicals like 2,3,5-triiodobenzoic Acid (TIBA) or high concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) induce genes involved in the first (eg. cytochrome *CYP81D11*) or second phase (eg. *GSTU7*) of the detoxification process. This induction strictly requires the TGA factors and SCL14 with evidence being provided by corresponding knockout mutants (Fode et al., 2008). The activation mechanism of the TGA/SCL14 complex remains to be understood.

1.2 Role of phytohormones in defense responses

Phytohormones are small molecules within plants that are crucial for growth, development, reproduction and survival. The hormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are the well-studied classical players of defense response mounted against different pathogens. In addition to these, the role of other phytohormones in defense is also emerging. Reports indicate that abscisic acid (ABA) (Asselbergh et al., 2008; Mauch-Mani and Mauch, 2005; Xu et al., 2013a), gibberellic acid (GA) (Yang et al., 2012), brassinosteroids (BR) (Albrecht et al., 2012; Nakashita et al., 2003), auxin (Navarro et al., 2006; Spaepen and Vanderleyden, 2011; Wang et al., 2007) and cytokinins (CK) (Naseem and Dandekar, 2012; Siemens et al., 2006) modulate the immune response.

1.2.1 Salicylic acid pathway

The phenolic signaling compound SA plays a major role in plant immune responses against biotrophic pathogens and is involved in pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) (An and Mou, 2011; Glazebrook, 2005; Loake and Grant, 2007; Pieterse et al., 2009; Tsuda et al., 2009; Vlot et al., 2009). It is also essential for establishing long-term immunity in the form of systemic acquired resistance (SAR) (Durrant and Dong, 2004).

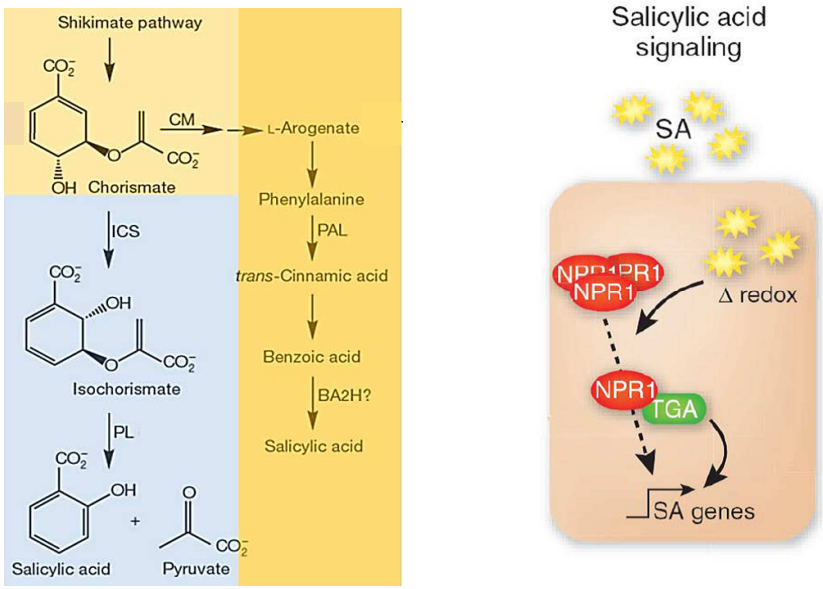


Figure 1.2 : Salicylic acid biosynthetic (adapted from Wildermuth et al., 2001) and signaling (Pieterse et al., 2009) pathways in plants

(Left) The isochorismate synthase (ICS) pathway (blue) is the primary route for SA production upon pathogen attack while the phenylalanine ammonia-lyase (PAL) pathway (yellow) has been implicated to play a minor role in *Arabidopsis thaliana*. Enzymes in the biosynthetic pathways are abbreviated as follows: isochorimate synthase (ICS), benzoic acid 2-hydoxylase (BA2H), pyruvate lyase (PL, identified in bacteria), chorismate mutase (CM). **(Right)** Simplified model of SA signaling suggests that SA accumulation changes the redox potential within the cell, resulting in reduction of the NPR1 oligomer to its active monomer state. NPR1 then gets translocated into the nucleus where it functions as a transcriptional co-activator with TGA transcription factors to induce SA-responsive genes

Biochemical and genetic approaches have revealed two pathways for the synthesis of SA in plants (Figure 1.2), the phenylalanine ammonia lyase (PAL)-pathway and the isochorismate synthase (ICS)-pathway (Chen et al., 2009; Wildermuth et al., 2001). Studies have indicated that the bulk of SA (~95%) that accumulates during plant-pathogen interaction is produced via the ICS pathway (Garcion et al., 2008). Once synthesized, SA can undergo modifications such as glucosylation, methylation or amino acid (AA) conjugation that make SA inactive and occur to fine-tune its accumulation or activity (Dempsey et al., 2011). SA signaling is mediated by both NPR1 (*NON EXPRESSOR OF PR GENES 1*) -dependent (Bowling et al., 1997; Cao et al., 1997; Dong, 2004; Shah et al., 1997) and NPR1-independent mechanisms (Bowling et al., 1997; Desveaux et al., 2004; Lorrain et al., 2004). In the un-induced state, NPR1 exists as an oligomer in the cell cytosol. Cellular redox changes result in its monomerization followed by its translocation to the nucleus where it is targeted for proteasomal degradation to dampen the basal expression of defense genes. SA perception stabilizes NPR1 in the nucleus which then acts as a co-activator for TFs like the TGA factors to regulate SA-responsive genes like the *PATHOGENESIS RELATED-1* (*PR-1*) (Fu et al., 2012; Lu, 2009; Mou et al., 2003; Spoel et al., 2009; Tada et al., 2008). However studies indicate that *PR* genes can be induced in an NPR1-independent manner indicating that there may be other proteins that also perceive SA (Blanco et al., 2005; Gou et al., 2009). Recently, it was found that the clade I TGA TFs (TGA1 and TGA4) positively regulate MAMP-triggered immunity through NPR1-independent mechanisms (Shearer et al., 2012; Wang and Fobert, 2013).

1.2.2 Jasmonic acid pathway

Jasmonates are lipid-derived molecules that regulate diverse processes like pollen maturation and response to wounding, herbivory and defense against necrotrophic pathogens (Browse, 2005; Wasternack, 2007). The biosynthesis of JA (Figure 1.3) begins with the octadecanoid pathway that initiates in the chloroplast when α-linolenic acid is released from membrane lipids by phospholipases (Ellinger et al.,

2010; Hyun et al., 2008; Wasternack, 2007). Further enzymatic reactions involving 13-lipoxygenases (13-LOX) (Caldelari et al., 2011; Chauvin et al., 2013), ALLENE OXIDE SYNTHASE (AOS) (von Malek et al., 2002; Park et al., 2002a) and ALLENE OXIDE CYCLASE (AOC) (Stenzel et al., 2003a, 2003b, 2012) give rise to 12-oxo-phytodienoicacid (OPDA) which travels to the peroxisome where it may be actively taken up via transporters (Theodoulou et al., 2005). OPDA is then converted to (+)-7-iso-JA through a series of reactions including three rounds of β-oxidation (Breithaupt et al., 2009; Kienow et al., 2008; Kombrink, 2012; Schilmiller et al., 2007; Stintzi and Browse, 2000). JA is subject to enzymatic conversions giving rise to numerous metabolites including amino acid conjugates [(+)-7-iso-Jasmonoyl-L-isoleucine (JA-Ile) being the natural bioactive jasmonate], methyl and glucose esters, hydroxylation products, the decarboxylation product cis-jasmone and reduced derivatives (Glauser et al., 2008, 2010; Kramell et al., 2005; Matthes et al., 2010; Seo et al., 2001; Staswick, 2009; Staswick and Tiryaki, 2004; Wasternack and Hause, 2013).

JA-perception and signaling is mediated via the F-box protein CORONATINE INSENSITIVE 1 (COI1) (Xie et al., 1998). The SCF^{COI1} complex targets the JASMONATE ZIM DOMAIN (JAZ) proteins for degradation allowing the release of positive activating TFs such as MYC2 that drives the expression of JA-responsive genes (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007). It was recently shown that the JAZ proteins also recruit co-repressors TOPLESS (TPL), TPL-related proteins (TPRs) through adaptor protein Novel interactor of JAZ (NINJA) (Pauwels and Goossens, 2011; Pauwels et al., 2010). MYC2 activates its own expression as well as that of JA-responsive genes like *VSP2* and *LOX2* which are known to be induced in response to herbivorous insects and mechanical wounding (Lorenzo et al., 2004; Reymond et al., 2000; Rojo et al., 1999; Turner et al., 2002).

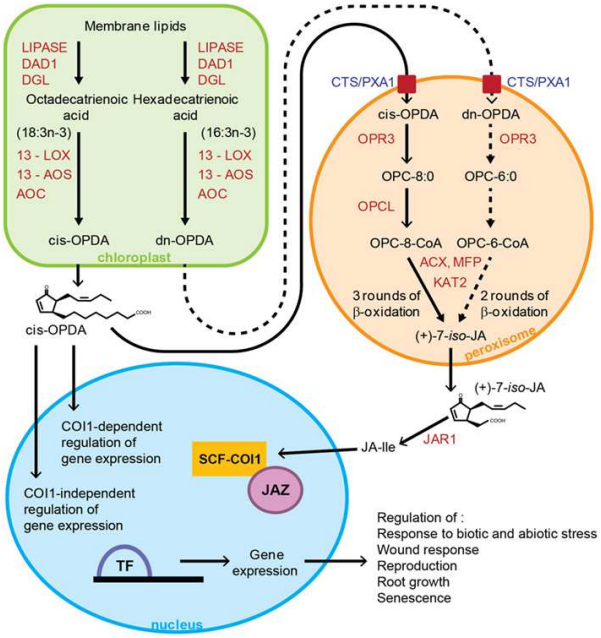


Figure 1.3: Jasmonic acid biosynthetic and signal transduction pathways in plants (Dave and Graham, 2012)

JA biosynthesis initiates in the plastid with intermediate cis-OPDA and dn-OPDA being transported into the peroxisome where sequential reactions lead to the formation of (+)-7-iso-JA. JAR1 catalyzes the formation of JA-Ile from JA in the cytosol which is perceived by the JA-receptor COI1 that then targets the JAZ repressors for degradation thus de-repressing the activation of JA-responsive genes. Enzyme names are shown in red. Dashed arrows indicate route to JA biosynthesis via dn-OPDA, where these steps are yet to be proven experimentally. DAD1, DEFECTIVE IN ANTHER DEHISCENCE1; DGL, DONGLE; 13-LOX, 13-lipoxygenase; 13-AOS, 13-allene oxide synthase; AOC, allene oxide cyclase; OPR3, 12-oxophytodienoate reductase3; OPCL1, OPC-8:CoA ligase1; CTS, COMATOSE; ACX, acyl CoA oxidase; KAT, 3-l-ketoacyl-CoA-thiolase; MFP, multifunctional protein; JA, jasmonic acid; cis-OPDA, cis-(+)-12-oxo-phytodienoic acid; dn-OPDA, dinor-oxo-phytodienoic acid; JA-Ile, jasmonoyl-L-isoleucine; COI1, CORONATINE-INSENSITIVE1; JAZ, jasmonate ZIM domain.

A second branch, leading to expression of JA-responsive genes like *PLANT DEFENSIN1.2* (*PDF1.2*) and *b-CHI* is regulated by a positive interaction between JA and ethylene (ET) through transcription factor *OCATDECANOID-RESPONSIVE*

ARABIDOPSIS 59 (*ORA59* (Berrocal-Lobo et al., 2002; Lorenzo et al., 2004; Penninckx et al., 1998)). Thus, the JA pathway seems to be branched into two pathways that are regulated by MYC2 and ORA59, the latter requiring both JA and ET (Zander et al., 2010). Recently it was shown that ET-stabilized ETHYLENE INSENSITIVE 3 (EIN3) and EIN3-LIKE1 (EIL1) interact directly with JAZ proteins which along with co-repressors inhibit transcriptional activity of EIN3/EIL1 (Zhu et al., 2011). Introduction of JA in the system induces COI1-mediated degradation of JAZ proteins thus relieving the repressive effect of JAZ proteins on ET signaling.

1.2.3 Antagonism between different phytohormones

When plants perceive a pathogen or "attack" they respond by activating specific defense responses which affect the accumulation patterns of different phytohormones. The ensuing hormonal blend, known as "signal signature", varies in strength, composition and timing depending on the lifestyle, invasion and plant-attacker combination (De Vos et al., 2005). Although there are exceptions, it is generally believed that pathogens with a biotrophic mode of lifestyle activate the SA-dependent immune responses while the necrotrophic pathogens and herbivorous insects stimulate a JA-dependent response (Glazebrook, 2005; Howe and Jander, 2008; Pieterse et al., 2012). These two hormones are the major players in plant immunity and it is well established that antagonism between the two plays a central role in modulating the immune response (Figure 1.4). Recently, it was shown that SA-mediated suppression of JA-responsive genes is downstream of the SCF^{COI1}-JAZ machinery and works by targeting GCC-box motifs found in JA-responsive promoters via negative effect on the transcriptional activator ORA59 (Van der Does et al., 2013). Another player in the SA-JA cross-talk is the SA-induced NPR1 which is required for suppressing the JA response (Leon-Reyes et al., 2009). Other players in the SA-JA antagonism include WRKY factors (e.g. WRKY70), MITOGEN ACTIVATED PROTEIN KINASE 4 (MPK4) and glutaredoxins (e.g GRX480) (Li et al., 2004; Ndamukong et al., 2007; Petersen et al., 2000). It has been shown that GRX480 and several other CC-type GRXs can interact with class II TGA factors and

suppress the JA/ET branch of JA-signaling by suppressing expression of *ORA59* (Ndamukong et al., 2007; Zander et al., 2012). The two branches of JA-signaling – MYC2 and ERF – themselves are antagonistic to each other (Figure 1.4). The JA-ET antagonism is modulated by an interaction between MYC2 and EIN3/EIL1 (Song et al., 2014). MYC2 can repress the EIN3/EIL1 to inhibit ET-regulated apical hook formation and response to necrotrophic pathogens. Conversely, EIN3/EIL can attenuate MYC2 to inhibit wound-responsive and herbivore-inducible gene expression.

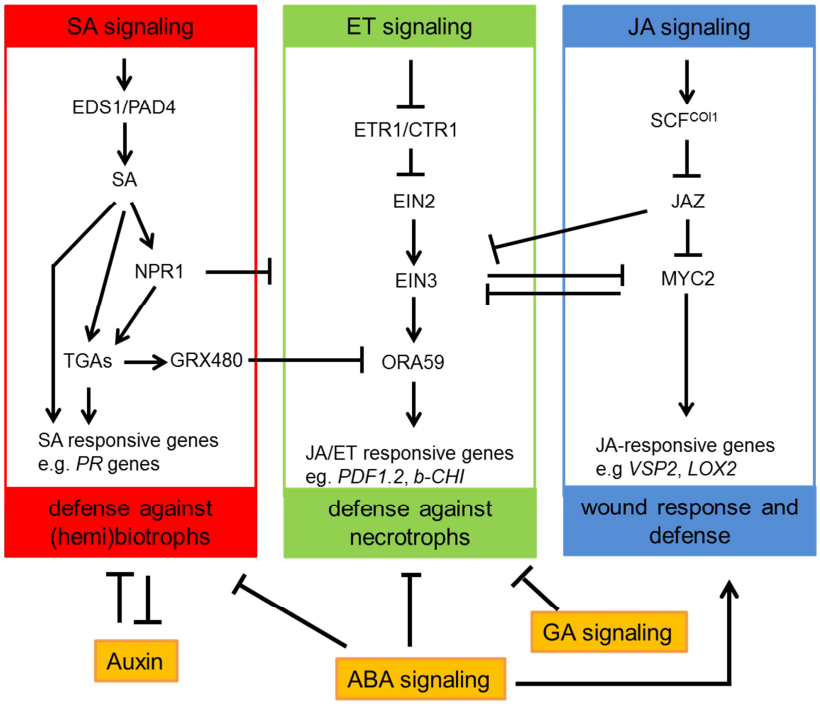


Figure 1.4: Cross-communication between phytohormones in immune response (adapted from Pieterse et al., 2009; Song et al., 2014; Zhu et al., 2011)

Cross-communication between hormone signaling pathways provides the plant with a large regulatory capacity that may tailor its defense response to different types of attackers. The SA, JA and ET signaling pathways represent the backbone of the defense signaling network, with other hormonal signaling pathways feeding into it. ⊥ indicates negative effect; arrows indicate positive effect.

As presented in Figure 1.4, abiotic stress and growth-related hormones like abscisic acid (ABA), gibberellic acid (GA), auxins and cytokinins usually feed into the defense network characterized by SA, JA and ET pathways to maintain the trade-off between defense and development (Gimenez-Ibanez and Solano, 2013; Naseem and Dandekar, 2012; Robert-Seilaniantz et al., 2011). Studies with external application of ABA suggest mainly a negative role of ABA in plant immunity including suppression of SA-mediated *PR-1* expression (Moeder et al., 2010). Similarly, *Arabidopsis* mutants impaired in ABA biosynthesis or sensitivity show more resistance to *Pst* DC3000 (de Torres-Zabala et al., 2007) as well as to necrotrophic fungal pathogen *Fusarium oxysporium* (Anderson et al., 2004). Making use of ABA and JA/ET signaling mutants it was shown that the two pathways act antagonistically to each other (Anderson et al., 2004). However ABA also plays a positive role in some instances where it was shown to contribute to JA accumulation and activation of JA-dependent resistance (Adie et al., 2007). The observations that ABA can antagonistically interact with defense pathways suggest that plant abiotic stress can be prioritized over plant immunity and suggests a central role for ABA in this cross-talk between biotic and abiotic stress (Cao et al., 2011; Mauch-Mani and Mauch, 2005).

1.3 Local and systemic wound response in plants

Although JA has been accepted as the wound hormone in land plants, our understanding of how it exerts its local and systemic effects is still incomplete. In tomatoes, many components other than JA were identified as signals leading to activation of wound-responsive genes. These included oligosaccharides (Bishop et al., 1984; Doares et al., 1995), the peptide systemin (Pearce et al., 1991), phytohormones ABA and ET (O'Donnell et al., 1996; Peña-Cortés et al., 1995; Weiss and Bevan, 1991) and electrical (Wildon et al., 1992) signals. It was believed however that all of them contributed to a single pathway operating through JA. It is now well established that there exists, in *Arabidopsis*, JA-dependent as well as JA-independent wound signaling pathways, even though the latter ones are not well

understood (León et al., 1998; McConn et al., 1997; Nishiuchi et al., 1997; Rojo et al., 1998, 1999; Titarenko et al., 1997). For example, it was shown that induction of *CHOLINE KINASE* (*CK*) and *WOUND-RESPONSIVE 3* (*WR3*) is independent of JA synthesis and perception (León et al., 1998; Titarenko et al., 1997). These genes were induced by oligosaccharides that are released from plant cell walls upon wounding and were shown to be specifically involved in the activation of JA-independent wound-induction pathway (Rojo et al., 1999). The same study proposed a model where the oligosaccharide-induced pathway suppressed JA-dependent wound responses in the local tissue via induction of ET biosynthesis [known to be activated in wounded tissue; (O'Donnell et al., 1996)] while proposing that JA was involved mainly in the systemic response after wounding. However, another report showed that the JA-responsive *AOS* gene was strongly expressed in both local and systemic leaves after wounding (Kubigsteltig et al., 1999). More recently it was shown that JA levels increase within 2-5 min of wounding throughout the plant (Glauser et al., 2008). Electric signals may be involved in transmitting this rapid signal for accumulation of JA in distal unwounded leaves (Mousavi et al., 2013; Wildon et al., 1992) and among the 13-LOXs, LOX6 seems to contribute to this rapid JA synthesis (Chauvin et al., 2013). Furthermore, there is evidence of another gene, *RNS1,* which accumulates in both wounded and systemic leaves but independently of both JA and oligosaccharide-mediated responses (LeBrasseur et al., 2002). These studies indicate that the wound response is much more complex than was presumed and although JA seems to play a major role, other signaling pathways may interact functionally to optimize the wound response.

1.4 NAC transcription factors

Over 5% of the *Arabidopsis* genome encodes for more than 2000 transcription factors and a sizeable percentage of these are specific to the plant kingdom. One such plant-specific group is the family of NAC transcription factors that are among the largest TF families in *Arabidopsis* consisting of more than a hundred members (Riechmann et al., 2000). The NAC (**N**AM/**A**TAF1/2/**C**UC2) domain was first

identified from the *NAM* (***N****O* ***A****PICAL* ***M****ERISTEM*) sequence from Petunia (Souer et al., 1996) and *ATAF1, ATAF2* (***A****RABIDOPSIS* ***T****HALIANA* ***A****CTIVATION* ***F****ACTOR 1, 2*) and *CUC2* (***CU****P-SHAPED* ***C****OTYLEDON*) sequences from *Arabidopsis* (Aida et al., 1997, 1999). Since then several members have been identified and characterized in *Arabidopsis* as well as in different plants like rice (Hu et al., 2006, 2008), soybean (Hao et al., 2011; Le et al., 2011; Tran et al., 2009), wheat (Kawaura et al., 2008; Xia et al., 2010), potato (Collinge and Boller, 2001) and even in tree species like poplar (Hu et al., 2010) and citrus (Liu et al., 2009; Oliveira et al., 2011). Few have also been reported in the moss *Physcomitrella patens* but none have been found in algae so far suggesting they are specific to land plants (Shen et al., 2009).

1.4.1 Structure

The NAC TFs are characterized by the presence of a consensus sequence, termed as NAC domain which is situated in the N-terminal region of the protein and includes the DNA binding domain (DBD) (Duval et al., 2002; Kikuchi et al., 2000). This N-terminal region consisting of approximately 150 amino acids is highly conserved as opposed to the vastly variable C-terminal end (Ernst et al., 2004; Olsen et al., 2005). The NAC domain consists of five sub-domains A-E [Figure 1.5 (i)]. The sub-domains A and B show a net negative charge while all other sub-domains are positively charged which may help in DNA binding (Ernst et al., 2004; Xie et al., 1999). Studies suggest dimerization of NAC proteins is possible via conserved interactions including salt bridges and that the sub-domain A plays a major role in this. The DBD is suggested to be contained within the sub-domains C and D while B and E sub-domains which are more diverse than others may contribute to functional diversity (Chen et al., 2011; Ernst et al., 2004; Jensen et al., 2010; Ooka et al., 2003). Structure of the NAC domain also reveals a unique transcription fold not yet seen in other TFs. It contains a twisted β-sheet surrounded by a few helical elements rather than the classical helix-turn-helix motif (Ernst et al., 2004). The transcriptional regulatory (TR) domain of the NAC proteins is usually present in the C-terminal end and can function to activate (He et al., 2005; Lu et al., 2007; Puranik et al., 2011; Tran et al., 2004) or to repress (Delessert et al., 2005; Kim et al., 2007; Yamaguchi et

al., 2010) transcription. The TR region being highly variable may contribute to the functional diversity seen among the NAC proteins. Further, there are also some NAC proteins that show variations from the above described typical structure which is described in Figure 1.5 (ii) – (vi) (Hao et al., 2010; Jensen et al., 2010; Mitsuda et al., 2004; Seo et al., 2008; Yoshiyama et al., 2009).

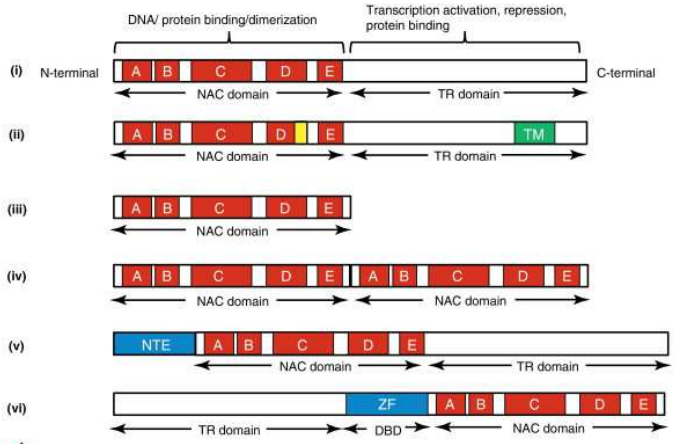


Figure 1.5: Structure of NAC proteins (Puranik et al., 2012)

Schematic representation showing (i) a typical NAC protein with a N-terminal NAC domain and a C-terminal transcriptional regulatory (TR) domain, (ii) NAC domain that may contain negative regulatory domain (shaded yellow) and/or a C-terminus having a transmembrane motif (shaded green), (iii) NAC proteins encoding only a NAC domain, (iv) proteins containing two tandemly repeated NAC domains, (v) proteins having a N-terminally extended (NTE) region preceding the conserved NAC domain and (vi) VOZ proteins that have a NAC domain at the C-terminal end while their TR domain lies at the N-terminal with a zinc-finger (ZF) motif functioning as a DBD (DNA-binding domain)

1.4.2 Classification

A decade ago, the first systematic analysis of NAC proteins was carried out with 75 and 105 predicted NAC proteins in rice and *Arabidopsis* respectively (Ooka et al., 2003). The proteins were classified into two supergroups (I and II) based on the predicted NAC sub-domain (A-E) amino acid sequences. The two groups were further divided into several sub groups on the basis of the NAC domain structure. With the identification of more than 100 putative NAC genes in rice, a new phylogenetic analysis (based on sub-domains A-D) was carried out which divided the

family into five major groups (1-5) (Fang et al., 2008). A further classification of NAC genes (based on sub-domain A-C) from tobacco and compared with rice, *Arabidopsis* and poplar revealed a new NAC sub-family named as TNACS that appeared to be restricted to the Solanaceae family since it has been identified so far only in tomato, potato and pepper (Rensing et al., 2008). Thus classification of the NAC TFs has been a challenge since phylogeny changes with the inclusion of different sub-domain sequences.

More recently, Shen et al., 2009 included the highly diverse C-terminal sequences of the NAC proteins and attempted to classify this complex TF family. A total of 1,232 NAC genes from eleven different organisms were included in a sequence based phylogeny analysis (based on N-terminal protein sub-domains A-E) which classified the proteins into eight subfamilies (NAC-a to NAC-h) (Figure 1.6). Each subfamily was further divided into subgroups based on tree topology and each subgroup members were divided into different clades based on their C-terminal motif patterns. The analysis showed that the C-terminus also show conserved motif patterns across sub-groups. Based on this the NAC genes with known different functions clearly fell into different subfamilies.

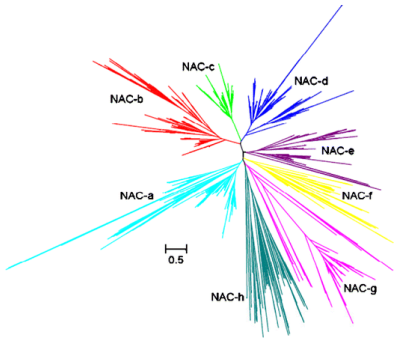


Fig 1.6 :Maximum likelihood (ML) phylogeny of 1,232 NAC proteins (Shen et al., 2009)

The NAC domain regions A-E were used in the analysis. The 1,232 proteins include 1,211 NAC proteins from 11 plant species (*Physcomitrella patens* spp *patens* (moss), *Selaginella moellendorffii* (spike moss), *Populus trichocarpa*, *Vitis vinifera* (grape), *Medicago truncatula*, *Glycine max*, *Oryza sativa*, *Sorghum bicolor*, *Zea mays*, *Panicum virgatum* and *Arabidopsis thaliana*) and an additional 21 NAC proteins collected from literature. Bar shows the distance scale for branch length (amino acid substitutions per site)

Earlier classification placed wound-responsive *ATAF1* and *ATAF2* in the ATAF subfamily along with *StNAC* and *OsNAC6* which are also stress-induced. This provided support to the idea that the members of the ATAF subgroup share a conserved role in response to stress stimuli (Ooka et al., 2003). In the new classification, biotic and abiotic stress related NAC proteins group into the NAC-a subfamily (Shen et al., 2009). The Arabidopsis ATAF sub-family members *ATAF1*, *ATAF2*, *ANAC032* and *ANAC102* contain the same motif clade and get grouped into NAC-a-sc2 clade which is part of the NAC-a-9 subgroup (Figure 1.7)

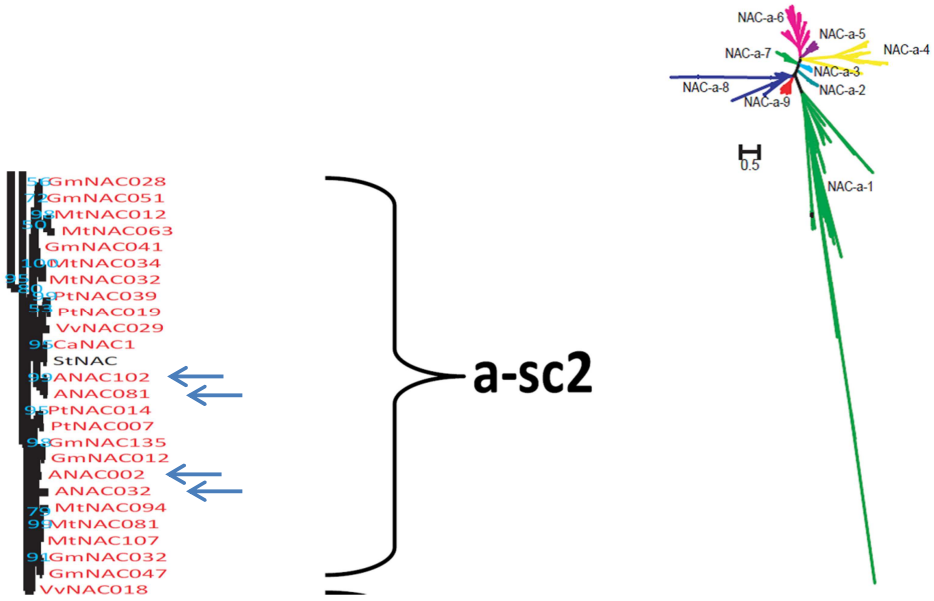


Figure 1.7: The motif clades and subgroups for the NAC-a subfamily.

Subgroups are a-1 to a-9 while motif clades are a-sc1 to a-sc20. The previously described ATAF subfamily members fall under a-sc2 clade (**left**) in sub-group NAC-a-9 (**right**) according to new classification. Blue arrows indicate the four *Arabidopsis* ATAF members (Shen et al., 2009, modified).

1.4.3 Biological functions of NAC proteins

Studies on *NAM* and *CUC2*, the first *NAC* genes to be characterized, suggested that these are essential for proper development because mutations in these genes resulted in fused cotyledons and a lack of shoot apical meristems causing the

mutants to die at a seedling stage (Aida et al., 1997; Souer et al., 1996). Since then a lot of studies in different plant species have provided information about the role of NAC proteins in plant growth and development (Aida et al., 1999; Hao et al., 2011; Hendelman et al., 2013; Hibara et al., 2006; Ishida et al., 2000; Kunieda et al., 2008; Ohtani et al., 2011; Pei et al., 2013; Ricachenevsky et al., 2013; Takeda et al., 2011). Many NAC proteins have also been identified by their induction in response to biotic stress. The potato *NAC* gene *StNAC* is rapidly induced in response to infection by *Phytophthora infestans* and wounding (Collinge and Boller, 2001). The *TaNAC8* and *GRAB1* from wheat are induced in response to stripe rust pathogen and wheat dwarf geminivirus infections respectively (Xia et al., 2010; Xie et al., 1999). Studies indicated that membrane-bound NTL6 is proteolytically activated by cold further leading to expression of *PATHOGENESIS-RELATED* (*PR*) genes which gave rise to the proposal of an adaptive strategy that protects plants against infection by hydrophilic pathogens known to occur in cold season (Seo et al., 2010a, 2010b). Recently, it was shown that infection with fungal *Verticillium* pathogen leads to induction of *VND7* (a NAC-domain protein) that regulates *de novo* xylem formation thus enhancing water storage capacity of the host plant (Reusche et al., 2012). The previously known wound-responsive *ATAF2* was shown to interact with Tobacco Mosaic Virus replicase protein which led to suppression of basal host defenses and promotion of systemic virus accumulation (Wang et al., 2009a). Over-expression of the closely related member *ATAF1*, also known to be induced after wounding, led to negative regulation of defense responses against necrotrophic pathogens in two independent studies, although its effect upon the expression of defense genes *PR1* and *PDF1.2* was contradictory in the two reports (Wang et al., 2009b; Wu et al., 2009). There is also controversial evidence regarding the role of *ATAF1* in abiotic drought stress situation. One study found *ataf1* mutant to be more drought tolerant and showed that ATAF1 negatively regulates expression of drought-responsive genes like *COR47*, *ERD10* and *RD29A* (Jensen et al., 2008; Lu et al., 2007). Another study reported that over-expression of *ATAF1* conferred increased drought tolerance in *Arabidopsis* along with a higher expression of *COR47* and *RD29A* genes at late stages of stress (Wu et al., 2009). One reason for these discrepancies may be

environmental factors that modulate cross-talks between different signaling pathways. The ATAF1 and ATAF2 TFs have also roles in ABA and auxin synthesis respectively by directly regulating expression of key biosynthetic enzymes (Huh et al., 2012; Jensen et al., 2013). Several other NAC TFs have been described as being induced under a variety of abiotic stress situations like osmotic stress, salinity, drought, cold and oxidative stress (Fujita et al., 2004; Hu et al., 2008; Irsigler et al., 2007; Jin et al., 2013; Ramegowda et al., 2012; Wu et al., 2009; Xu et al., 2013b). Thus, more and more studies are emerging that indicate the diverse roles played by NAC TFs.

Most knowledge of potential downstream target genes of NAC TFs arise from transcriptome analyses in plants overexpressing the NAC protein. One study identified CGT(G/A) and CACG as core-DNA binding motif recognized by drought-responsive NAC TFs ANAC019, ANAC055 and ANAC072 (Tran et al., 2004). Another study involving a calmodulin-binding NAC protein identified GCTT as the core NAC binding sequence flanked by other frequently repeating sequences (TT**GCTT**ANNNNNNAAG) (Kim et al., 2007). More recently, the ATAF1 consensus binding sequences were identified as T(A/C/G)CGT(A/G) and TT(A/C/G)CGT (Jensen et al., 2013). Another analysis which made use of the recent ChIP-Seq and RNA-Seq techniques identified three potential NAC binding motifs [G(A/T/C/G)G(A/G)G(A/G)G(A/G); C(A/C)C(G/A)(T/C/G)(G/A)C(C/G) and TGGGCC] for NAC proteins that are specifically induced during development of soybean seedlings (Shamimuzzaman and Vodkin, 2013). Future studies may reveal other novel NAC recognition sequences improving our understanding of functional diversity of NAC proteins.

1.4.4 Regulation of NAC proteins

In spite of extensive functional characterization of so many different NAC proteins, their regulation is poorly understood. Transcriptional regulation of stress responsive NAC TFs has been postulated by the presence of several stress-responsive *cis*-acting elements in promoter regions. These include the ABA-responsive elements (ABREs), low-temperature responsive elements (LTREs), MYB and MYC binding

sites, W-box, JA-responsive elements and SA-responsive elements (reviewed in Olsen et al., 2005; Puranik et al., 2012). A few NAC TFs have been shown to be regulated post-transcriptionally by microRNAs (miRNAs). For example, the *NAC1* gene in maize was negatively regulated by *miR164b* (Li et al., 2012) and the *AsNAC60* in creeping bentgrass was shown to be regulated by *miR319* (Zhou et al., 2013). A further mode of regulation of NAC TFs can be post-translational including ubiquitin-mediated protein degradation (Greve et al., 2003; Xie et al., 2002), dimerization (Ernst et al., 2004; Jeong et al., 2009; Puranik et al., 2012) and interactions with other proteins (Greve et al., 2003; Tran et al., 2007). The nuclear import of NTLs (membrane associated NAC TFs) is regulated by proteolytic cleavage of the anchor by transmembrane proteases, often in response to different stresses. An example is the NTL6 which is cleaved and imported to the nucleus under cold stress (Seo et al., 2010a). Another example of post-translational regulation is that of ATAF1 which was shown to interact with SnRK1 kinases *in vitro*. This interaction could modulate either the DNA binding activity of ATAF1 or target ATAF1 to proteasomal degradation by a second interaction of SnRK1 with E3-like ligases (Kleinow et al., 2009).

2. Aim of the Study

This work is based on the findings of Dr. Benjamin Fode (PhD dissertation). It was shown that class II TGA factors (TGA2, TGA5 and TGA6) recruit the GRAS family protein SCL14 (SCARECROW-like 14) to promoters of downstream target genes. This TGA256/SCL14 complex triggers the detoxification response against toxic chemical substances like TIBA (2, 3, 4-triiodobenzoic acid). Microarray analysis revealed a number of genes that were up-regulated when *SCL14* was overexpressed. The promoter sequences of these genes were then scanned for *as-1*-like elements to which TGA factors can bind (Katagiri et al., 1989). Among the candidate target genes of the TGA/SCL14 complex that were thus identified, two of them belonged to the ATAF sub-family of NAC transcription factors (TFs) (Fode et al., 2008). These two members – *ANAC032* and *ATAF1* – are close homologs and along with other two members of the ATAF sub-family (*ANAC102* and *ATAF2*) show similar expression profiles in response to a wide range of treatments (Kleinow et al., 2009). Although, *ATAF1* was among the first NAC transcription factors to be identified (Souer et al., 1996), its function is not yet well understood. In this current thesis, we have tried to comprehend the possible functions of ATAF-type NAC TFs with more focus on *ANAC032* as it has not been characterized before.

Since *ANAC032* and *ATAF1* were identified as targets of the TGA/SCL14 complex, the primary aim of the current study was to understand their contribution to the detoxification response and identify potential downstream target genes in the pathway. For this purpose, transgenic plants over-expressing the two NAC TFs as well as knockout mutants of the two were obtained and analyzed.

Further, the ATAF subfamily of TFs is predicted to be stress-responsive (Ooka et al., 2003). There has been contradicting evidence regarding the possible roles of *ATAF1* in biotic as well as abiotic stress responses (Mauch-Mani and Flors, 2009). ATAF1 has been suggested to positively as well as negatively regulate gene expression of defense genes like *PR1* (Wang et al., 2009b; Wu et al., 2009). In a previous study

(Dr. Julia Köster, PhD dissertation) it was observed that over-expression of *ANAC032* in *Arabidopsis* resulted in a suppression of methyl jasmonate (MeJA)-induced *CYP81D11* and *PDF1.2* expression. These evidences suggest some role for the two NAC TFs in defense responses. It is known that phytohormones like SA and ABA can act antagonistically with JA/ET leading to suppression of *PDF1.2* (see Introduction 1.2.3). Since the cross-talk depends on TGA factors and because these hormones can induce the NAC TFs, a role for the NAC proteins in mediating these cross-talks is also explored.

Transgenic over-expression lines of *35S:ANAC032* and *35S:ATAF1* that were generated showed developmental phenotypes as compared to the wild-type plants. Additionally, a look at the transcriptomic database, Arabidopsis eFP browser, indicated high levels of *ANAC032* in the seed stage compared to any other plant tissue. Consequently, we have investigated their role in seed dormancy and also its contribution to development and growth of *Arabidopsis*.

To summarize, the key questions addressed in this current thesis were as follows:

1. Do *ANAC032* and *ATAF1* regulate a part of the TGA256/SCL14-induced response to xenobiotic compounds? If yes, what could be the down-stream target genes?

2. Which role do the NAC TFs play during the defense response involving the phytohormones? Do these TFs, as speculated from previous reports, regulate cross-talks seen frequently between different biotic and/or abiotic stress signaling pathways?

3. What is the probable role of *ANAC032* in the development and growth of *Arabidopsis*?

3. Materials and Methods

3.1 Materials

3.1.1 Organisms

3.1.1.1 Bacteria

Strain	Description	Reference
Agrobacterium tumefaciens GV3101::pMP90	Rif^r, Gent^r	(Koncz and Schell, 1986)
Escherichia coli DB3.1	F– *gyr*A462 *end*A1 Δ(sr1-*rec*A) *mcr*B *mrr* *hsd*S20(rB–, mB–) *sup*E44 *ara*-14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20(SmR) *xyl*-5 λ– *leu* *mtl*1	(Bernard and Couturier, 1992)
Escherichia coli DH5α	F– Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1	(Hanahan, 1985)

3.1.1.2 Fungi

Strain	Description	Reference
Botrytis cinerea (named as BMM strain)	Infects *A. thaliana Col-0*	Kindly provided by Brigitte Mauch-Mani, University of Neuchatel, Switzerland
Saccharomyces cervisiae (PJ69-4a strain)	MATα, trp1-901, leu2-3,112, ura3-52, his3-200, gal4Δ, gal80Δ, GAL2-ADE2, LYS2 ::GAL1-HIS3, met2::GAL7-*lac*Z	(James et al., 1996)

3.1.1.3 Insect

Species	Reference
Plutella xylostella (Diamondback moth)	Kindly provided by Department of Agricultural Entomology, University of Göttingen, Germany

3.1.1.4 Plant (*Arabidopsis thaliana*)

Genotype	Description	Reference
35S:ANAC032	Transgenic line over-expressing (N-terminal) HA-tagged *ANAC032* gene under the control of the *CaMV 35S* promoter	Dr. Julia Köster; personal communication

35S:ATAF1	Transgenic line over-expressing (N-terminal) HA-tagged *ATAF1* gene under the control of the *CaMV 35S* promoter	Dr. Julia Köster; personal communication
anac032 (SALK_012253)	T-DNA insertion in the first exon of *ANAC032* gene	NASC stock no. N512253
ataf1-1 (SALK_067648)	T-DNA insertion in the third exon of the *ATAF1* gene	(Lu et al., 2007)
ataf1anac032	Obtained by crossing knockout mutants *ataf1-1* and *anac032*	Dr. Julia Köster; personal communication
coi1-t	T-DNA insertion within the *COI1* locus	(Mosblech et al., 2011)
Columbia accession-0 (*Col-0*)	Wild type (WT)	NASC stock no. N1092
delayed-dehiscence2-2 (dde2-2)	Knockout mutation in ***A****LLENE* ***O****XIDE* ***S****YNTHASE* (*AOS*) gene	(Park et al., 2002b)
scl-14 scl-33 (*scl14/33*)	Double knockout of *SCL14* and *SCL33* obtained by crossing T-DNA insertion mutants of each gene	Alexander Meier; personal communication
tga2-1 tga5-1 tga6-1 (*tga256*)	Combined deletion knockout mutants of *TGA2*, *TGA5* and *TGA6*	(Zhang et al., 2003)
JAZ10-GusPlus	JA-responsive reporter construct wherein *JAZ10* promoter and 5'UTR region is cloned upstream of the GUSPlus™ gene	(Acosta et al., 2013)

3.1.2 Media and Buffers

3.1.2.1 Media

Media	Composition
dYT	2% tryptone 1% yeast extract 1%NaCl
Luria Bertani (LB)	1% tryptone 0.5% yeast extract 1% NaCl (1.5% agar in case of LB agar)
Murashige and Skoog (MS)	4.4g/L MS (Duchefa Chemie) pH 5.7 with KOH 8g/L Select Agar (Invitrogen)
Potato Dextrose broth (PDB)	Sigma-Aldrich, Germany
Selective amino acid dropout -Leu/ -Trp (SD-LT)	0.67% yeast ammonia base without amino acids 2% glucose 0.062% SD -Leu, -Trp, -Ura

	20mg/L Uracil pH 5.6 with 1M NaOH (1.2% Select agar for SD-LT agar plates)
Yeast extract-peptone-adenine-dextrose (YPAD)	1% yeast extract 2% peptone 2% glucose 10mg Adeninehemisulfate pH 6.0 with HCl (1.2% Select agar in case of YPAD agar plates)
YEB	10g beef extract 2g yeast extract 5g peptone 5g saccharose pH 7.0 with 5N NaOH after autoclaving sterile 2mM $MgSO_4$ was added

3.1.2.2 Buffers and Solutions

Media	Composition
Buffer I for alkaline lysis	50mM Tris-HCl (pH 8.0) 10mM EDTA 100µg/µl RNase A
Buffer II for alkaline lysis	0.2M NaOH 1% (w/v) SDS
Buffer III for alkaline lysis	3M potassium acetate 5% formic acid
DNA extraction buffer	200mM Tris-HCl (pH 5.7) 250mM NaCl 25mM EDTA (pH 8.0) 0.5% SDS
Enzyme solution for protoplast isolation	1.25% cellulose 0.3% macroenzyme 0.4M mannitol 20mM KCl 20mM MES (pH 5.7) 10mM $CaCl_2$
Fixing solution (for GUS staining protocol)	50mM sodium phosphate buffer (pH 7.0) 0.3 M mannitol 0.3% formaldehyde
GUS extraction buffer	50mM sodium phosphate buffer (pH 7.5) 10mM EDTA 0.1% Triton X-100 0.1% Sarkosyl Freshly add 5µl β-mercaptoethanol/10ml buffer just before use
GUS Stop solution	0.2M Na_2CO_3
High Salt Precipitation Buffer (HSPB)	1.2M NaCl 0.8M tri-sodium-citrate
MEN (10X)	200mM MOPS 50mM NaOAc 10mM EDTA pH adjusted to 7.0 with 1M NaOH
MMg solution	0.4M mannitol 15mM $MgCl_2$

	4mM MES (pH 5.7)
MUG solution	4mM MUG (4-methyl-umbelliferyl-ß-D-glucuronide) dissolved in GUS extraction buffer
ONPG solution	4mg/ml o-nitrophenyl-β-D-galactopyranoside; dissolved in Z-buffer
ONPG Stop Solution	1M Na_2CO_3
PEG solution for protoplast transformation	20g PEG4000 13.3ml 0.75M mannitol 5ml 1M $CaCl_2$, Make volume up to 50ml using sterile de-ionized water
RNA loading buffer (3X)	100µl bromophenol-blue 80µl 0.5M EDTAp (pH 8.0) 333µl 10X MEN 1200µl glycerol (100%) 4286µl formamide 1001µl formaldehyde Add 6µl EtBr/ml loading buffer right before use
TAE (1X)	40mM Tris-acetate 1mM EDTA (pH 8.0)
Trizol buffer	380ml/L phenol with 0.1M Citrate buffer pH4.3 saturated 0.8M guanidine thiocyanate 0.4M ammonium thiocyanate 33.4ml/L 3M sodium acetate 5% glycerin
W5 solution	154mM NaCl 125mM $CaCl_2$ 5mM KCl 2mM MES (pH 5.7)
WI solution	0.5M mannitol 4mM MES (pH 5.7) 20mM KCl
X-Gluc Staining solution	50mM sodium phosphate buffer (pH 7.0) 0.5mM potassium ferricyanide 0.5mM potassium ferrocyanide 2.5mM bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) 10mM EDTA 2% dimethyl formamide 1% Triton X-100
Yeast transformation mix (prepared fresh before use)	240µl 50% (w/v) PEG3500 36µl 1M lithium acetate 50µl Salmon Sperm DNA (previously boiled at 95°C for 10min) 29µl sterile water
Z-buffer	60mM $Na_2HPO_4,7H_2O$ 40mM NaH_2PO_4,H_2O 10mM KCl 1mM $MgSO_4$ pH 7.0

3.1.3 Antibiotics

Antibiotic	Stock solution	Working concentration	Source
Gentamycin	25mg/ml in water	25mg/L	Duchefa
Kanamycin	50mg/ml in water	50mg/L	Sigma
Rifampicin	10mg/ml in water	50mg/L	Duchefa
Spectinomycin	50mg/ml in water	100mg/L	Sigma-Aldrich

All stock solutions were prepared and stored at -20°C

3.1.4 Hormones and Chemicals

Hormone/Chemical	Concentration	Source
1-aminocyclopropane-1-carboxylic acid (ACC)	1mM in de-ionized H_2O	Sigma
2,3,4-triiodobenzoic acid (TIBA)	100µM in DMSO	Sigma-Aldrich
Abscisic acid (ABA)	100µM in 0.1%EtOH	
Methyl jasmonate (MeJA)	4.5µM	Sigma-Aldrich
Salicylic acid (SA)	1mM in de-ionized H_2O	Merck

3.1.5 Kits, Enzymes and Standards

Kit/Enzyme/Standard	Supplier
Advantage 2 Polymerase Mix	Clonetech
BioTaq DNA Polymerase	Bioline
BP Clonase™ II Enzyme Mix	Invitrogen
DNAse I	MBI Fermentas
Dual Luciferase Reporter Assay System	Promega
GeneRuler 1kb DNA ladder	MBI Fermentas
iProof High-Fidelity DNA Polymerase	Bio-Rad
LR Clonase™ II Enzyme Mix	Invitrogen
Nucleobond® PC 500	Macherey-Nagel
Nucleospin Extract II	Macherey-Nagel
Nucleospin Plasmid	Macherey-Nagel
Restriction Enzymes	MBI Fermentas
Reverse Transcriptase	MBI Fermentas
RNase A	Qiagen
RNeasy Plant Mini Kit	Qiagen

3.1.6 Plasmids

Plasmid	Description	Reference/Source
pB2GW7	GATEWAY™ vector for plant transformation, contains CaMV 35S promoter and BASTA	(Karimi et al., 2002)

	resistance gene as selection marker, Spec^r	
pB2GW7-HA	pB2GW7 vector with an additional N-terminal 3X HA-tag	Dr. Corinna Thurow, personal communication
pB2GW7-HA-ANAC032	pB2GW7-HA derivative containing the *ANAC032* coding sequence	Dr. Julia Köster, personal communication
pB2GW7-HA-ATAF1	pB2GW7-HA derivative containing the *ATAF1* coding sequence	Dr. Julia Köster, personal communication
pBGWL7	Gateway™ vector for plant transformation, contains the firefly (ff) luciferase reporter gene, and a BASTA resistance gene as selection marker, Spec^r	(Karimi et al., 2002)
pBGWL7-3g04000prom	pBGWL7 derivative containing 1267bp promoter fragment (-1248 to +19 region) of *At3g04000* gene	This thesis
pBGWL7-AKR4C9 prom	pBGWL7 derivative containing 939bp promoter fragment (-913 to +26 region) of *AKR4C9* gene	This thesis
pBGWL7-bHLH585prom	pBGWL7 derivative containing 1250bp promoter fragment (-1205 to +45 region) of *At1g10585* gene	This thesis
pBGWL7-FLCprom	pBGWL7 derivative containing 2709bp promoter fragment (-2537 to +133 region) of *FLC* gene	This thesis, Ulla Schneider (Master student)
pDONR201	GATEWAY™ -adapted entry vector to generate attL flanked entry clones containing gene of interest following recombination with an attB expression clone or PCR product, Km^r	Invitrogen
pDONR201-ANAC032	pDONR201 derivative containing the coding sequence of *ANAC032* gene	Dr. Julia Köster, personal communication
pDONR201-ATAF1	pDONR201 derivative containing the coding sequence of *ATAF1* gene	Dr. Julia Köster, personal communication
pDONR207	GATEWAY™ -adapted	Invitrogen

	entry vector to generate attL flanked entry clones containing gene of interest following recombination with an attB expression clone or PCR product, Gm^r	
pDONR207-3g04000prom	pDONR207 derivative containing 1267bp promoter fragment of *At3g04000* gene	This thesis
pDONR207-AKR4C9prom	pDONR207 derivative containing 939bp promoter fragment of *AKR4C9* gene	This thesis
pDONR207-bHLH585prom	pDONR207 derivative containing 1250bp promoter fragment of *At1g10585* gene	This thesis
pDONR207-FLCprom	pDONR207 derivative containing 2709bp promoter fragment of *FLC* gene	This thesis, Ulla Schneider (Master student)
pUBQ10GW7-HA	GATEWAY™ vector for plant transformation, contains *UBQ10* promoter and BASTA resistance gene as selection marker, $Spec^r$	Li-Jun Huang; personal communication
pUBQ10GW7-HA-VP16	VP16 fragment was cut out of the vector pALLIGATOR1 with *Kpn*2I and ligated into pUBQ10GW7-HA	This thesis, Ulla Schneider (Master student)
pUBQ10-HA-ANAC032	pUBQ10-HA derivative containing coding sequence of *ANAC032* gene	This thesis
pUBQ10-HA-ANAC032-VP16	pUBQ10GW7-HA-VP16 derivative; contains coding sequence of *ANAC032* and VP16 domain at its C-terminal end	This thesis, Ulla Schneider (Master student)
pUBQ10-HA-ATAF1	pUBQ10-HA derivative containing coding sequence of *ATAF1* gene	This thesis
pUBQ10-HA-ATAF1-VP16	pUBQ10GW7-HA-VP16 derivative; contains coding sequence of *ATAF1* and VP16 domain at its C-terminal end	This thesis, Ulla Schneider (Master student)

3.1.7 Oligonucleotides

QuantiTect Assay Primer (QPA) was ordered from Qiagen. All other primers were ordered from Invitrogen (Life Technologies)

3.1.7.1 Oligonucleotides for qRT-PCR

Primer	Sequence (5'-3')
AKR4C9	QPA (QT00718732*)
ANAC032	QPA (QT0074356*)
ANAC102	QPA (QT00848582*)
At1g10585 (bHLH585)	QPA (QT01124690*)
At3g04000	QPA (QT00726054*)
ATAF1fwdRT	GAG TTC ACG AGC GAG GTT CA
ATAF1revRT	TCC ACG GTG GCA TCA ATG TA
ATAF2	QPA (QT00825769*)
COR78	QPA (QT00840406*)
FLC	QPA (QT00826448*)
PDF1.2 RT fwd	CTT GTT CTC TTT GCT GCT TTC
PDF1.2 RT rev	CAT GTT TGG CTC CTT CAA G
PR1 fwd	CTG ACT TTC TCC AAA CAA CTT G
PR1 rev	GCG AGA AGG CTA ACT ACA ACT AC
UBQ5 fwd	GAC GCT TCA TCT CGT CC
UBQ5 rev	GTA AAC GTA GGT GAG TCC A
VSP2 fwd RT	CAA ACT AAA CAA TAA ACC ATA CCA TAA
VSP2 rev RT	GCC AAG AGC AAG AGA AGT GA

*Qiagen QuantiTect Primer catalog number

3.1.7.2 Oligonucleotides for sequencing or genotyping

Primer	Sequence (5'-3')
ATAF1_RP	TAA AAC GGT CTC GTG TTG CCA TAA
ATAF1_UP	CGC CAA GTT TCA GAG GTA GAG AGA G
LBb1 (for SALK line genotyping)	GCG TGG ACC GCT TGC TGC AAC T
SALK_012253_RP	CTT AAT ACC AAC CGG TTT AGG ACG
SALK_012253_UP	TTT TTA ATT ACG GCG GAA AGA GAA TAG
Seq-L1	TCG CGT TAA CGC TAG CAT GGA TCT C
Seq-L2	GTA ACA TCA GAG ATT TTG AGA CAC

3.1.7.3 Oligonucleotides for cloning

Primer	Sequence (5'-3')
3g0400prom_fwd	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTG GTT CAT GGA TTT GCT CCG C
3g0400prom_rev	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TGA TGA CGC TGC AGC CAT
AKR4C9prom_low	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG CCA TTA TCA GAT GTG GTG GT
AKR4C9prom_up	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC CTA AGA CTA TTT CCT TAG TTC GCG

bHLH585prom_low	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC CCA TTC TTT TTG TTT TCT TTT AAG CT
bHLH585prom_up	GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC TAG TCA CGG ATT TTC AAT GGC TA
FLCprom_fwd	GGG GAC AAG TTT GTA CAA AAA GCA GGC TTA TTC GTG TTG CAA AAT CG
FLCprom_rev	GGG GAC CAC TTT GTA CAA GAA AGC TGG GTG CCA TGG CTT CTC TCC GAG AGG

3.1.8 Instruments

Instrument/Consumable	Supplier
arium® pro DI Ultrapure Water System	Sartorius Stedim Biotech
Centro XS^3 LB 960 DLReady™ Luminometer	Berthold Technologies
Cooling microcentrifuge	Hettich Lab Technology
Gel electrophoresis unit EV2xx	© Consort bvba
Gene Pulser® II	BioRad
Heraeus Pico17 micro centrifuge	Thermo Scientific
MyCycler™ thermocycler	BioRad
Nanodrop 2000	PeqLab Biotechnologies
pH meter HI 2212	Hanna Instruments
Photometer Libra S11	Biochrom
Real-time PCR iCycler	BioRad
ROTINA 38R Ultracentrifuge	Hettich Lab Technology
Synergy HT Plate reader	BioTek Instruments

3.1.9 Software

Software	Supplier
AgriGO tool	(Du et al., 2010)
Bio-Rad iQ5	Bio-Rad
Clone Manager ver 7	Sci-Ed Software
Geneious 5.3	Biomatters Limited
GraphPad Prism 5	GraphPad Software
Oligo 4.0	Molecular Biology Insights. Inc

3.2 Methods

3.2.1 Plant material, growth conditions and treatments

Arabidopsis thaliana (accession *Columbia* [*Col-0*]) was used as wild type in this study. The overexpressing lines *35S:ANAC032* and *35S:ATAF1* were maintained as heterozygous seed batch and homozygous plants were selected for all the experiments based on the size of the plant (Homozygous *35S:ANAC032* and *35S:ATAF1* plants are extremely dwarf in comparison to *Col-0* (see Results section 4.8 and Figure 4.18). Sterilized *Arabidopsis* seeds were grown either on sterile agar plates containing Murashige and Skoog (MS) medium or on soil under environmentally controlled conditions. Vapour-phase sterilization of the seeds was carried out using solution of 100ml sodium-hypochlorite and 5ml concentrated HCl.

For germination assays, sterilized seeds were sown on sterile MS medium containing 100µM TIBA or 0.1% DMSO (as control). The plates were stratified at 4°C in the dark for two days before shifting them to long day conditions (**LD**; 22°C, 16-h light/8-h dark cycle, 60% relative humidity and light intensity of 100-150µmol/m^2s).

For soil grown plants, sterilized seeds were sown on autoclaved soil and kept at 4°C for two days for stratification. They were then grown under LD or short day (SD; 22°C, 8-h light/16-h dark cycle, 60% relative humidity and light intensity of 100-150µmol/m^2s) conditions depending on the treatment as mentioned below. For all treatments (except for wounding and *Botrytis* infection) the complete rosette was harvested.

3.2.1.1 TIBA treatment

Six-week old plants, grown in controlled climate chambers under SD conditions, were sprayed with 100µM TIBA or 0.1% DMSO and material was harvested 8 hours later and immediately frozen in liquid nitrogen.

3.2.1.2 MeJA treatment

Plants were grown in controlled climate chambers under LD conditions. Four-week old plants were placed in a closed glass aquarium (sealed with wax) where 4.5µM MeJA was applied via the gaseous phase with help of Whatman filter paper. Plant material was harvested 8 hours later and immediately frozen in liquid nitrogen.

3.2.1.3 ACC treatment

Four-week old plants grown under LD conditions were sprayed with 1mM ACC or with de-ionized water as mock treatment. Material was harvested 24 hours later and immediately frozen in liquid nitrogen. (Treatment NOT done in glass aquarium)

3.2.1.4 SA treatment

Four-week old plants grown under LD conditions were placed in a closed glass aquarium (sealed with wax) and sprayed with 1mM SA for 24 hours after which material was harvested and immediately frozen in liquid nitrogen.

3.2.1.5 ABA treatment

Plants were grown under LD conditions and four-week old plants were placed in a glass aquarium (sealed with wax) and sprayed with 100µM ABA. Material was harvested either 8 hours later or 24 hours later as required and immediately frozen in liquid nitrogen.

3.2.1.6 Ozone treatment

Four-week old LD grown plants were placed in the ozone cabinet where ozone levels were maintained at 0.3ppm for 6 hours after which the plants were harvested and frozen in liquid nitrogen. As control, plants were placed in a similar cabinet for 6 hours but without ozone supply.

3.2.1.7 Wounding

Leaf surface of four-week old LD or 12-h light/12-h dark cycle grown plants were wounded with the help of a forcep. Wounded leaf material was harvested 90 minutes after wounding and immediately frozen in liquid nitrogen. To analyze systemic and local response, 2-3 leaves per rosette were wounded which were collected as local tissue sample and un-wounded leaves in the same rosette were collected as systemic tissue sample.

3.2.1.8 *Botrytis cinerea* infection studies

Six-week old plants grown under controlled environmental conditions with a 12-h light/12-h dark cycle were used for *Botrytis cinerea* infection assays. *Botrytis cinerea* (strain BMM) was grown on potato dextrose agar (PDA) plates kept in darkness at 20-22°C until full sporulation occurred. Harvesting of the spores was then done by adding quarter strength potato dextrose broth (PDB) onto the plates and then filtering through Mira cloth (Calbiochem®). Number of spores was counted under the microscope using a counting chamber and then was diluted in PDB to get a final concentration of $5X10^6$spores/ml. For infection, plants were placed inside a glass aquarium filled with little water at the bottom to maintain humidity. Leaf surface of 5-6 leaves per plant was spot-inoculated with 6µl of $5X10^4$spores/ml *Botrytis* culture. Quarter strength PDB was used to spot-inoculate surfaces of control plants. The diameters of lesions were measured three days post infection (dpi) and infected leaves were harvested by freezing in liquid nitrogen.

3.2.1.9 Insect feeding assay

Four-week old plants grown under LD conditions were placed in a closed glass aquarium (NOT sealed with wax) and one larva (1st instar of *Plutella xylostella*) was placed on top of each plant. The insect were allowed to feed on the plants until it had eaten approximately half of the plant leaf tissue.

3.2.2 Molecular biology methods

3.2.2.1 Genomic DNA isolation from *Arabidopsis* (Edwards et al., 1991)

Sample was collected using the lid of a 1.5ml centrifuge tube to pinch out a disc of material (from 2-3 week old plant) and immediately frozen in liquid nitrogen. The frozen material was crushed inside the centrifuge tube itself using a pestle after which 400µl of DNA extraction buffer [200mM Tris-HCl (pH 7.5), 250mM NaCl, 25mM EDTA (pH 8.0) and 0.5%SDS] was immediately added to it. The sample was then centrifuged for 5 min (13000rpm, RT) and 300µl of the supernatant was taken into a new centrifuge tube containing 300µl of isopropanol. Additional centrifugation step was carried out for 5 min (13000rpm, RT) and the resulting pellet was washed with 70% EtOH by a final centrifugation step of 5 minutes (13000pm, RT). The pellet was then dried at 37°C for 10 min and dissolved in 100µl de-ionized water. The concentration of isolated DNA was determined using the Nanodrop 2000 spectrophotometer.

3.2.2.2 Plasmid DNA isolation from *E. coli*

Plasmid DNA isolation from *E. coli* was prepared either using commercially available kits or by alkaline lysis method, depending on purpose. The concentration of isolated plasmid DNA was determined using Nanodrop 2000 spectrophotometer.

For sequencing and cloning purpose, high purity plasmid DNA was isolated using the Nucleospin Mini kit (Macherey and Nagel). A 5ml O/N culture was always used and plasmid was eluted in 50µl de-ionized water. Larger amounts of plasmid DNA was prepared using the NucleoBond™ PC 500 Kit (Macherey and Nagel). Final volume was decided based on the size of the DNA pellet to be re-suspended in de-ionized water.

Small amounts of plasmid DNA for analytical purposes were isolated using a modified alkaline lysis method (Le Gouill et al., 1994). 2ml of an overnight *E. coli* culture was centrifuged for 1 min (13000rpm, RT) and the supernatant was discarded. The cells were re-suspended in 100µl of Buffer I (50mM Tris-HCl (pH 8.0), 10mM EDTA, 100µg/µl RNase A) by vortexing. To this 200µl of Buffer II (0.2M NaOH, 1% (w/v) SDS) was added and the suspension was incubated on ice for 5 min. Buffer II was neutralized by addition of 150µl of Buffer III (3M CH3COOK, 5% formic acid) and inverting the tubes 6-8 times. The suspension was then centrifuged for 10 min (13000rpm, RT) after which the aqueous supernatant was transferred to a new tube containing 1ml 96% (v/v) EtOH. The DNA was left to precipitate at -20°C for 20 min. The precipitated DNA was collected by centrifugation for 10 min (13000rpm,

4°C). The pellet obtained was washed with 70% EtOH and dried at 37°C for 10 min. The DNA pellet was then dissolved in 20µl de-ionized water.

3.2.2.3 Restriction digestion of DNA

The digestion reactions were incubated in a buffer system optimized for the used enzyme. The amount of enzyme (Enzyme Unit, U) necessary for each digestion reaction was determined according to following formula,

$$U = \frac{[\text{Lambda phage } (\lambda) \text{ DNA length in bp}] \times [\text{no. of RE sites in target sequence}]}{[\text{No. of RE sites in } \lambda \text{ sequence}] \times [\text{size of target sequence in bp}]}$$

3.2.2.4 Agarose Gel Electrophoresis

Analysis and separation of DNA molecules was done by electrophoresis using an agarose gel matrix. The gel was run horizontally with 1X TAE (40mM Tris-acetate and 1mM EDTA, pH 8.0) as the running buffer. For DNA fragments between 500-14000bp, samples were run on 1% agarose whereas for lower size DNA fragments a 2%agarose gel was used. The DNA samples were mixed with 1/10th volume of 10X DNA Loading Buffer and then loaded into separate lanes in the cast gel. Electrophoresis was then carried out at 120V for ~45 min. After the run, the gel was stained using 0.1% (w/v) ethidium bromide and then visualized under a UV trans-illuminator (Gel Doc system). The size and amount of DNA fragments were determined using a DNA standard (DNA ladder mix, MBI Fermentas, Germany).

The elution of DNA fragments from agarose gel for cloning purpose was carried out using the Nucleospin Extract II Gel Extraction kit (Macherey-Naggel, Germany)

3.2.2.5 PCR

Polymerase chain reaction (PCR) was performed to amplify oligonucleotide of interest for cloning purposes or a 3-primer PCR was performed for genotyping T-DNA insertion lines. PCR reactions were carried out in a MyCycler™ Bio-Rad thermocycler. For cloning purposes, the iProof™ High Fidelity DNA polymerase (Bio-Rad) was used with denaturation and extension steps carried out at 98°C and 72°C respectively. The annealing temperature depended on the primers used. For PCR-based genotyping, the Advantage *Taq* DNA Polymerase was used along with genomic DNA as the template. The wild type allele was identified with the combination of RP and LP primers while the corresponding T-DNA insertion alleles was identified with the combination of the RP and LB primers. Homozygous mutant plants gave a single band corresponding to the use of RP and LB primers while heterozygous mutant plants gave two PCR fragments corresponding to both sets of primers.

3.2.2.6 Gateway® cloning

The GATEWAY® Technology with Clonase™ II system from Invitrogen was used for cloning binary vectors for transient and stable plant transformations. The technology makes use of the site-specific recombination properties of lambda phage and provides a rapid and efficient way to clone DNA sequences (flanked by modified *att* sites) into multiple vectors (Hartley et al., 2000).

3.2.2.6.1 BP reaction

For generating entry clones, PCR reaction (using iProof high-fidelity DNA polymerase) was carried out such that the gene of interest was flanked with modified *att*B sites (*att*B1: 5'-GGGG ACA AGT TTG TAC AAA AAA GCA GGC T-3' ; *att*B2: 5'-GGGG ACC ACT TTG TAC AAG AAA GCT GGG T-3'). Purified *att*B-PCR products were then used in a BP recombination reaction where equimolar amounts (~150ng) of the PCR product and of either pDONR201 (Km^r) or pDONR207 (Gm^r), along with 2µl of BP Clonase™ II enzyme mix was kept at RT for 1 hour. The reaction was then transformed into *E. coli* DH5α cells as described in 3.2.2.7

3.2.2.6.2 LR reaction

For creating expression clones, an LR reaction was carried out between an *att*L containing entry vector and an *att*R containing destination vector (pUBQ10HA or pBGWL7). Purified plasmid of the entry clone (50-150ng) was added to 150ng of the destination vector along with 2µl of LR Clonase™ II enzyme mix and kept at RT for 1 hour. The reaction was then transformed into *E. coli* DH5α cells as described in 3.2.2.7

3.2.2.7 Transformation of *E. coli* (Hanahan, 1985)

Heat shock was used to transform chemically competent *E. coli* DH5α cells that were prepared using the $CaCl_2$ method and stored at -80°C until use. 200µl of the competent cells were thawed on ice for 20-30 min before adding 50-70ng of plasmid DNA. The contents were mixed gently and then incubated on ice for 30 min. Heat shock was then applied by keeping the mixture at 42°C for 90 s after which the cells were immediately placed on ice for 5-10 min. 700µl of LB medium was added to the cells and the suspension was mixed on a horizontal roller for 60 min at 37°C. The culture was then spread on LB agar plates supplemented with antibiotic (as required for selection). The plates were incubated overnight at 37°C.

3.2.2.8 Transformation of *A. tumefaciens* (Mattanovich et al., 1989)

Cells of *A. tumefaciens* GV3101 strain (stored at -80°C prior to use) were transformed using the electroporation method. Cells were thawed on ice before adding 100-200ng plasmid DNA to it. The mixture was then transferred to an electroporation cuvette with an electrode distance of 0.2cm. A single electric pulse of 2.5kV initial voltage (25µF, 400W) was applied using GenePulser II. The cells were immediately suspended in 1ml YEB (1% beef extract, 0.2% yeast extract, 0.5%

peptone, 0.5% saccharose, pH 7.0, after autoclaving, sterile 2mM $MgSO_4$) medium and incubated for 2 h at 29°C. The culture was then spread on YEB agar plates supplemented with antibiotics (as required for selection). Plates were incubated for 2-3 days at 29°C.

3.2.2.9 Transformation of *Arabidopsis* (Clough and Bent, 1998)

Arabidopsis plants were transformed via *Agrobacterium*-mediated gene transfer using the floral dip method. Agrobacterium cells, transformed with gene of interest, were grown over-night in 20ml YEB medium supplemented with antibiotics (as required for selection) at 29°C on a shaker. This pre-inoculum was then used to inoculate 380ml of YEB medium (with antibiotics) and the culture was incubated overnight at 29°C with constant shaking. Cells were then harvested by centrifugation for 20 min (4000rpm, RT) and re-suspended in 200ml of 5% (w/v) saccharose solution. 100µl of Silwet surfactant was added and *Arabidopsis* inflorescence was dipped into the solution. Plants were then shifted back to the climate chambers and kept under humid conditions over-night. Positive T1 transformed plants were selected by BASTA selection.

3.2.2.10 Transformation of *Saccharomyces cervisiae* (Gietz and Woods, 2002)

A high efficiency transformation protocol was used to transfer PJ69-4a yeast strain in yeast-two-hybrid assays. The yeast cells were grown overnight in 20ml YPAD medium at 29°C on a shaker (200rpm). The optical density (OD) at 600nm was measured the following day using the Photometer Libra S11, Biochrom and enough cells were transferred to 80ml of YPAD medium such that the new OD would be ~0.4. The culture was then again incubated for 4-5 hours (for approx. two cell divisions to occur) at 29°C on a shaker. Cells were harvested by centrifugation for 5 min (4000rpm, RT) and resulting pellet was washed once with sterile water by centrifugation for 5 min (4000rpm, RT). The pellet was then re-suspended in 1ml of sterile water and distributed as 100µl aliquots into 1.5ml centrifuge tube (number of aliquots depend on number of transformation reactions). 355µl of transformation mix (240µl PEG 3500 50% (w/v); 36µl 1M LiAc; 50µl boiled ss carrier DNA; 29µl sterile de-ionized H2O) and plasmid DNA (bait + prey plasmid for yeast two hybrid, 500ng each). The mixture was incubated at 29°C for 30 min after which it was kept at 42°C for 40 min. The reaction mixture was then centrifuged for 30 s (13000rpm, RT) and the cell pellet obtained was re-suspended into 1ml sterile water and different dilutions were spread on selective SD-LT agar plates and incubated at 29°C for 2-3 days.

3.2.2.11 Sequencing

Samples were sent for sequencing to SeqLab-Microsynth AG, Göttingen. Samples were prepared as per the company's requirements.

3.2.2.12 RNA extraction (Chomczynski, 1993)

The TRIZOL extraction method was used to isolate RNA from plant material. 1.3ml of Trizol buffer (380ml/l phenol saturated with 0.1M citrate buffer pH 4.3, 0.8M

guanidine thiocyanate, 0.4M ammonium thiocyanate, 33.4ml 3 M Na-acetate pH 5.2, 5% glycerol) was added to frozen grinded plant material (100-200mg). After continuous vortexing for 15 min, 260µl chloroform was added to each sample and the tubes were vortexed for another 15 min. The samples were then centrifuged for 60 min (13000rpm, 4°C). 900µl of the supernatant was taken into a new microcentrifuge tube that contained 325µl precipitation buffer (HSPB, 1.2M NaCl, 0.8M Na-citrate) and 325µl 2-propanol. The contents were mixed by inverting the tubes several times and then incubated for 10 min at RT. After a second centrifugation step for 30 min (13000rpm, 4 °C), pellets were washed with 70% EtOH. After complete removal of EtOH, pellets were dried at 37°C for 5 min. The RNA pellet was then dissolved in 40-60µl of de-ionized water. The concentration of extracted RNA was determined using Nanodrop 2000 spectrophotometer.

3.2.2.13 cDNA preparation

cDNA was synthesized from 1µg of RNA. The RNA samples were initially incubated with 1µl DNase I (along with 1µl 10X DNase I buffer, Fermentas) for 30 min at 37°C. DNase I was then denatured by adding 1µl 25mM EDTA and incubating the samples for 10 min at 60°C. 20pmol of oligo dT primer and 200pmol of random nonamer oligonucleotides were added to the samples and the mixture was incubated for 10 min at 70°C. Finally, 20nmol dNTPs, 4µl RT 5X-Reaction Buffer and 60U Reverse Transcriptase H- (Fermentas) were added and the reaction mixture was incubated at 42°C for 30 min followed by incubation at 70°C for 10 min. The cDNA thus prepared was stored at -20°C.

3.2.2.14 Quantitative real-time PCR

Gene expression was analyzed using quantitative real-time PCR (qRT-PCR). Amplification and quantification was carried out in the iCycler System (Bio-Rad, USA). The reaction mixture consisted of 1X NH4-reaction buffer (Bioline, Germany); 2mM $MgCl_2$; 100µM of dNTPs; 0.4µM of primers (self-made or QuantiTect®, Qiagen), 0.25U BIOTaq DNA polymerase (Bioline, Germany); 10nM Fluorescein (Bio-Rad, USA); 100,000 times diluted SYBR Green I solution (Cambrex, USA); 1µl of 1:10 diluted cDNA as template. The obtained Ct values were normalized to housekeeping gene *UBQ5* and relative quantification was done using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

3.2.2.15 Microarray

For performing a microarray analysis, whole rosettes from 4-week old *Col-0* and *35S:ANAC032* plants (five individual plants as replicates) were harvested. The RNA extracted by Trizol method was purified using the RNeasy Plant Mini Kit (Qiagen) and samples was sent to Westfälische-Universität Münster, Integrierte Funktionelle Genomik (Germany) where the array was performed with *Arabidopsis* ATH1 genome arrays.

Functional enrichment of differentially regulated genes was analyzed by singular enrichment analysis (SEA) with the agriGO tool (Du et al., 2010). The statistical

method used was Fisher Test while the Yekutieli method was used for multiple comparison correction.

3.2.3 Protoplast isolation and transformation (Sheen, 2001)

Leaves from 3-4 week old plants (grown in 12h-light/12h-dark photoperiod) were used for isolation of protoplasts. The protocol followed for isolation and transformation was as described by Sheen, 2001. The isolated protoplasts were transformed with 5µg of promoter constructs (promoter of interest gene:firefly *luciferase* gene; pBGWL7 derivatives), 7.5µg of effector constructs (pUBQ10-HA derivatives) and 1µg of *35S:Renilla luciferase* plasmid. The firefly and renilla luciferase activities were measured the following day as described in 3.2.4

3.2.4 Luciferase assay

After over-night incubation, the transformed protoplasts settle at the bottom. The incubation buffer (WI solution, 33.3ml 0.75M mannitol, 2ml 0,5M KCl, 0.4ml 0.5M MES, volume made upto 50ml using de-ionized water) was then removed using a syringe and the sample was immediately frozen in liquid nitrogen. The luciferase assay was then performed using the Dual Luciferase Reporter Assay System from Promega. The measurements were recorded using the DLR Ready luminometer.

3.2.5 ONPG assay

Yeast PJ69-4a strain was transformed using bait and prey vectors (as described in 3.2.2.10). The yeast transformants were inoculated into 5ml of SD-LT medium and incubated overnight at 29°C on a shaker. On the following day, culture was centrifuged for 1min (13000rpm, RT) and supernatant was discarded. After washing the cell pellet once using 1ml Z-buffer (60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgSO_4$), they were re-suspended in 300µl of Z-buffer out of which 100µl was transferred to a new 1.5ml centrifuge safe-lock tube (rest of the solution was kept on ice for measuring OD of yeast cells). The cells were then lysed by repeated freezing and thawing using liquid nitrogen. After this 700µl of Z-buffer with 0.27% freshly added β-mercaptoethanol was added to the cell lysates and also to a tube containing 100µl of Z-buffer which served as a blank control. The contents were mixed by inverting the tubes and then 160µl of ONPG solution (4mg/ml *o*-nitrophenyl-β-D-galactopyranoside in Z-buffer) was added, mixed and reactions were incubated at 37°C until development of yellow colour. After colour development, time was recorded and reaction stopped using ONPG stop solution (1M Na_2CO_3). Samples were then centrifuged for 10 min (13000rpm, RT) and 200µl of the supernatant was added to wells of a microtitre plate. Amount of *o*-nitrophenyl was determined by measuring OD at 420nm using the BioTek plate reader. Dilutions of the cell suspension (previously stored on ice) were added to another microtitre plate and OD was measured at 595nm. The β-galactosidase activity were measured in units as,

$$\beta\text{-Gal units} = \frac{1000 \times OD_{420}}{T \times OD_{600} \times F}$$

where, T = reaction time in minutes
F = dilution factor for cell suspension

3.2.6 Metabolite measurements

Determination of various metabolite concentrations were performed using HPLC-MS/MS by the Department of Plant Biochemistry (Prof. Dr. Ivo Feußner, Dr. Tim Iven), University of Göttingen, Germany.

3.2.7 Quantitative GUS assay (Jefferson et al., 1987)

3.2.7.1 Protein extraction for MUG assay

Crude protein extracts were prepared by addition of 150µl GUS extraction buffer (50mM sodium phosphate buffer pH 7.5, 10mM EDTA, 0.1% Triton X-100, 0.1% Sarkosyl; freshly added 10mM β-mercaptoethanol) to frozen ground plant material. Samples were kept on ice till they thawed after which contents were mixed by inverting the tubes. The samples were then centrifuged for 10 min (10000rpm, RT). Clear supernatant was transferred to new 1.5ml centrifuge tubes and placed on ice.

3.2.7.2 Protein estimation

Protein concentrations were determined using a commercial Bradford assay solution (CARL ROTH, Germany). Equal volumes of protein extract (0.5-2µl) were added to wells of a microtitre plate containing 200µl of 5-fold diluted Bradford assay solution. After mixing the contents with pipette tips, the reaction was incubated at RT for 5-10min after which OD was measured at 595nm using the BioTek plate reader. Protein concentrations were determined with the help of standard curve derived from different known BSA concentrations (1, 3 and 6µg).

3.2.7.3 Measurement of relative GUS activity

Volume of protein extract, corresponding to approximately 25µg of protein, was added to well of flat-bottomed microtitre plate and filled up to 100µl using GUS extraction buffer. To this, 100µl of MUG solution (4mM MUG in GUS extraction buffer) was added and contents were mixed using pipette tips. The reaction was incubated at 37°C for 10 min in dark. 100µl of the sample was then transferred to a new well containing 100µl of GUS stop solution (0.2M Na2CO3); this served as t0 (zero time-point) value. The plate was further incubated at 37°C for 60 min in dark after which 100µl of GUS stop solution was added to the remaining reaction providing t60 values. Fluorescence was then measured at 365nm using the BioTek plate reader. Relative GUS activities were calculated from the t0 and t60 values.

3.2.8 GUS staining

Tissue to be analyzed (siliques or leaves) were taken in 2ml centrifuge tubes and submerged in the fixing solution (50mM sodium phosphate buffer (pH 7.0), 0.3M mannitol and 0.3% formaldehyde) for 30 min. After washing twice with 50mM sodium phosphate buffer (pH 7.0), 2 ml of X-Gluc staining solution (50mM sodium phosphate buffer (pH 7.0), 0.5mM potassium ferricyanide, 0.5mM potassium ferrocyanide, 2.5mM 5-Bromo-4-chloro-3-indolyl-β-D-glucuronide, 10mM EDTA, 2%

dimethylyformamide and 0.1% Triton X-100) was added. Samples were subjected to vacuum infiltration for 3 min and then incubated O/N at 37°C. Chlorophyll was then cleared by washing with 100% ethanol after which the GUS-stained samples were visualized under the microscope.

3.2.9 Juvenility assessment

The phenotypic analyses to distinguish juvenile and mature adult leaves was done as described by Willmann and Poethig, 2011. Juvenile leaves were defined as rosette leaves without abaxial (on the lower surface) trichomes, transition leaves as rosette leaves with abaxial trichomes that did not fully span the proximodistal axis, and adult leaves as rosette leaves with abaxial trichomes covering the whole surface up to the distal tip.

3.2.10 Germination test for seed dormancy analysis

Freshly harvested seeds from siliques that had turned slightly brown (but not yet dried) were collected for analysis of dormancy. Completely dried seeds fully released from dormancy (dried since more than 10-weeks) were used as controls. About 50 seeds (freshly harvested or completely dried out) were plated onto a filter paper moistened with demineralized water in Petri dishes and incubated under long day conditions. Scoring of radicle emergence was done 3 days later.

4. Results

4.1 *ANAC032* and *ATAF1* are downstream target genes of the TGA/SCL14 complex in the detoxification response

Fode et al., 2008 showed that *SCL14* (*SCARECROW-LIKE 14*) acts as a transcriptional co-activator of class-II TGA transcription factors (TFs). The TGA/SCL14 complex regulates the expression of genes involved in the detoxification of toxic chemical compounds. Whole-genome microarray analysis identified two NAC TFs – *ANAC032* and *ATAF1* – which showed more than a four-fold up-regulation in the *HA3-SCL14* overexpressing transgenic line as compared to the *scl14* knockout mutant. It was shown by chromatin immunoprecipitation assays that SCL14 is recruited to direct target genes by TGA factors (2, 5 and 6) which are known to bind to *as-1* (*activation sequence-1*)*-like* elements in promoter regions (Katagiri et al., 1989). Since the promoters of the two NAC factors contain putative *as-1-like* elements, they are likely to be direct targets of the TGA/SCL14 complex (Figure 4.1 A). To confirm that the NAC gene expression actually depends on the TGA/SCL14 complex under inducing conditions, wild-type, *tga256* [combined deletion knockout mutants of *TGA2*, *TGA5* and *TGA6*, (Zhang et al., 2003)] and *scl14/33* [combined T-DNA insertion mutants of *SCL14* and its close homologue *SCL33*] plants were treated with the xenobiotic toxic chemical TIBA (2,3,5-triiodobenzoic acid) and expression was analyzed. Quantitative real time-PCR (qRT-PCR) indicated that both, *ANAC032* and *ATAF1* are induced by application of TIBA and that this induction requires the presence of class-II TGA factors and SCL14/SCL33 (Figure 4.1 B). Moreover, the basal expression seemed to be slightly reduced in the *tga256* and *scl14/33* knockout plants. The two other closely related NAC proteins – *ATAF2* and *ANAC102* – belonging to the same motif clade as *ANAC032* and *ATAF1* (Supplementary Figure S1) - also contain *as-1-like* elements in their respective promoters (Figure 4.1 A). Consistently, these two TFs are induced by TIBA in a TGA/SCL14-dependent manner (Figure 4.2 B).

A

Gene	AGI code	*as-1-like* element (-1000 bp promoter region)
ANAC032	At1g77450	$_{-206}$ TGACGTAAgcaaTGACAAAA $_{-187}$
ATAF1/ANAC002	At1g01720	$_{-179}$ AGACGTAAgcaaTGACAACA $_{-160}$
ATAF2/ANAC081	At5g08790	$_{-231}$ TGACGCAAgcaaTGACAACA $_{-212}$
ANAC102	At5g63790	$_{-187}$ TGACGGAAgcaaTGACATCA $_{-168}$
consensus		TGACGTCAnnnnTGACGTCA

B

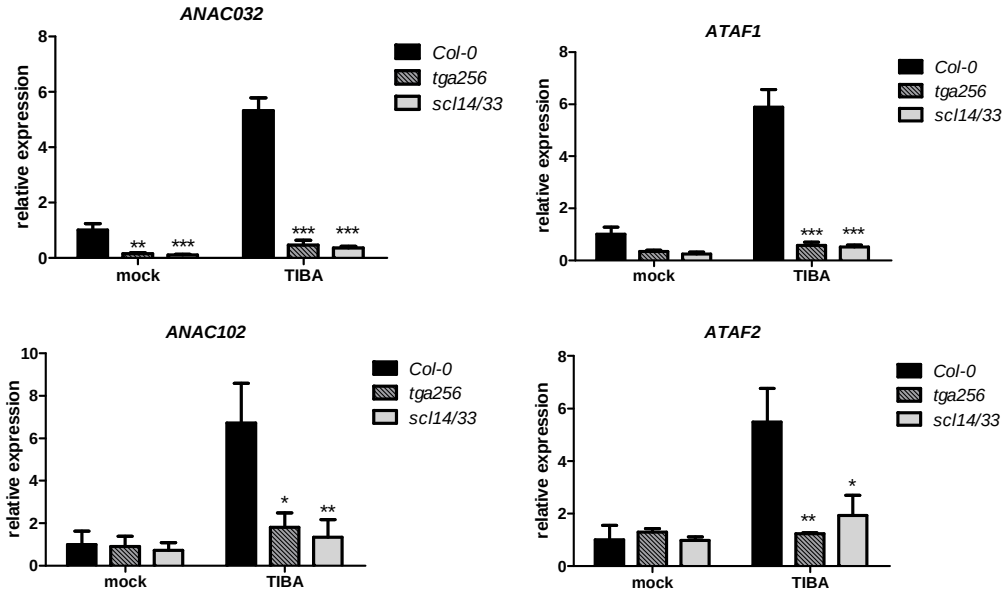


Figure 4.1: Expression of TIBA-induced ATAF TFs require the TGA/SCL14 complex

A: Table showing the sequences of putative *as-1-like* elements present in the promoter regions of the four ATAF-type TFs. The numbers indicate their positions relative to the transcriptional start sites (+1). Conserved nucleotides within the 8-bp palindromes (capital letters) are highlighted in red. The consensus sequence described for the *as-1* element (Katagiri et al., 1989) is shown in the last row.

B: Six-week old soil-grown (short day) *Col-0*, *tga256* and *scl14/33* plants were treated with 0.1mM TIBA for 8 hours. Treatment with 0.1% DMSO served as control (mock). The relative transcript levels (normalized to house-keeping gene *UBQ5*) of *ANAC032*, *ATAF1*, *ANAC102* and *ATAF2* were determined by qRT-PCR. The average relative expression in mock treated *Col-0* plants was set to 1. The mean values (±SE) from two experiments - each with 4-5 individual plants - are shown. Asterisks indicate significant differences compared with wild-type (Two-way ANOVA; ***P<0.001, **P<0.01, *P<0.05)

It was shown earlier that *tga256* and *scl14* display increased sensitivity towards TIBA (Fode et al., 2008). In a similar experiment, the NAC single knockouts *ataf1* and *anac032* (T-DNA insertion lines) and double knockout *ataf1anac032* were grown on MS plates containing TIBA to evaluate their sensitivity towards the toxic chemical. However, the single and double knockout plants behaved like the wild-type in their ability to germinate and grow on TIBA-containing media (Figure 4.2). This suggests that these two NAC TFs are not essential for the response towards TIBA and instead may be regulating just a sub-branch of the TGA/SCL14-triggered response. Alternatively, functional redundancy between the four NAC TFs in response to TIBA (Figure 4.1 B and Figure 4.6 A) may account for the absence of increased sensitivity in single and double knockouts.

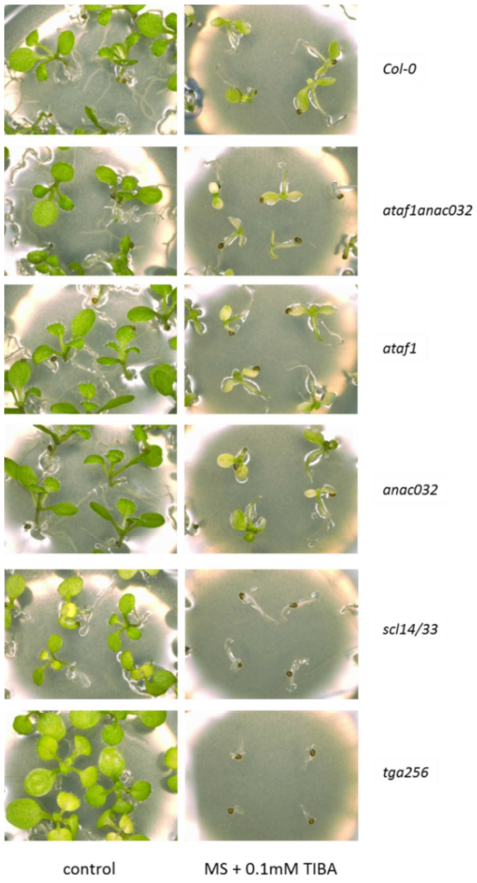


Figure 4.2: *ANAC032* and *ATAF1* is not essential for the observed sensitivity of *tga256* and *scl14/33* mutant plants towards the toxic chemical TIBA

Col-0, *ataf1anac032*, *ataf1*, *anac032*, *scl14/33* and *tga256* mutant plants were sown on MS plates containing 0.1mM TIBA and grown under long-day conditions. Photographs were taken two weeks after germination.

4.2 *AKR4C9, bHLH585* and *At3g04000* may be direct downstream targets of ANAC032 in the detoxification response towards xenobiotic compounds

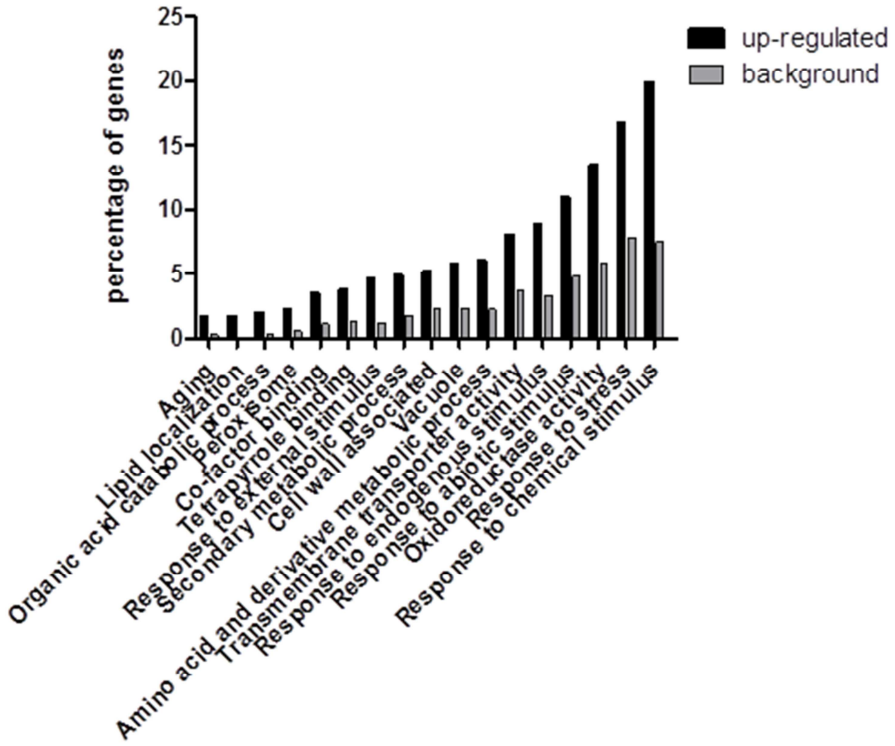
To further elucidate the role of the two NAC proteins in the detoxification response after TIBA treatment, transgenic plants ectopically expressing HA-ANAC032 or HA-ATAF1 under the control of the *Cauliflower mosaic virus (CaMV) 35S* promoter were generated. As mentioned above, SCL14-dependent genes containing *as-1-like* elements that were identified in the microarray analysis were assumed to be direct targets of the TGA/SCL14 complex (Fode et al., 2008). However there were a number of SCL14-dependent genes that contain no *as-1-like* element in their promoters. Therefore we speculated that these may be downstream genes of other TFs, like e.g. NAC proteins. To explore this and other possible target genes of the NAC TFs, microarray analysis, using RNA from untreated samples of *Col-0* and *35S:ANAC032* plants, was carried out. Of the 22,000 *Arabidopsis* genes on the Affymetrix ATH1 gene chip, 347 genes were more than 2-fold ($p<0.05$) up-regulated while 147 were more than 2-fold ($p<0.05$) down-regulated in the *35S:ANAC032* plants when compared with wild-type. The fold-induction of the top twenty up-regulated genes is shown in Table 4.1 (The complete list of significantly up- and down-regulated genes is listed in supplementary Table S1 and S2, respectively).

Table 4.1: List of twenty most up-regulated genes in *35S:ANAC032*

No.	Gene code	Symbol	Description	*35S:ANAC032 /Col-0*	p-value
1	AT1G77450	ANAC032	NAC domain containing protein	115.4942215	4.61E-09
2	AT2G37770	AKR4C9	aldo/keto reductase family protein	56.89304997	7.81E-07
3	AT1G10585		bHLH transcription factor	43.08498055	1.48E-06
4	AT3G26690	ATNUDX13	bis (5'-adenosyl)-pentaphosphatase/ hydrolase	29.7490688	3.83E-08
5	AT1G69880	ATH8	thioredoxin H-type 8	25.14220013	0.000349
6	AT1G66570	ATSUC7	sucrose-proton symporter	24.81858879	0.000148
7	AT4G23680		major latex protein-related / MLP-related	23.64446669	9.69E-05
8	AT1G62570	FMO GS-OX4	Flavin-Monooxygenase Glucosinolate S-Oxygenase 4	19.91268303	1.43E-06
9	AT1G02850	BGLU11	Hydrolase	19.66124129	2.18E-06
10	AT2G43820	SAGT1	Salicylic acid glucosyltransferase	18.93009073	7.38E-05
11	AT3G50970	LTI30	Low Temperature-Induced 30	15.20645997	0.000104
12	AT3G09260	PYK10	beta-glucosidase	14.52883705	0.005794
13	AT3G04000		short-chain dehydrogenase/ reductase protein	13.73017707	0.000384
14	AT1G34040		alliinase	13.44781841	1.23E-05
15	AT2G38380	PER22	peroxidase	13.32886285	0.001039
16	AT2G37760	AKR4C8	aldo/keto reductase family protein	12.63507277	2.64E-05
17	AT4G15490	UGT84A3	UDP-glycosyltransferase	12.35113466	8.21E-06
18	AT2G45210		auxin-responsive protein-related	11.79463674	1.19E-05
19	AT2G04040	ATDTX1	multidrug efflux pump	11.73727594	0.000732
20	AT5G10140	FLC	FLOWERING LOCUS C; transcriptional repressor	11.57218365	0.000308

The set of 494 differentially regulated (>2-fold, $p<0.05$) genes was further analyzed for specific enrichment of different gene ontology (GO) terms as specified by the TAIR database. As shown in Figure 4.3, several GO terms were significantly enriched when compared to the background (i.e. set of genes present in the ATH1 Affymetrix chip). Within the up-regulated genes (Figure 4.3 A), 30% were associated with response to different stimuli (abiotic, chemical, and biotic stress). Approximately 13% of up-regulated genes showed oxidoreductase activity and 8% were transmembrane transporters of which most belonged to the group of multidrug efflux transporters. Other significantly up-regulated GO terms included cell-wall, vacuole and peroxisome – associated, secondary metabolic process, amino acid metabolic process, organic acid catabolic process, glucosyl transferases and co-factor binding. Among the down-regulated genes only few GO terms were enriched (Figure 4.3 B). These were catalytic activity (45%; includes kinase, hydrolase and monooxygenase activity), response to stimulus (30%), endomembrane associated (28%), cell-wall associated (8%), oxygen and tetrapyrrole binding (5% each).

A



B

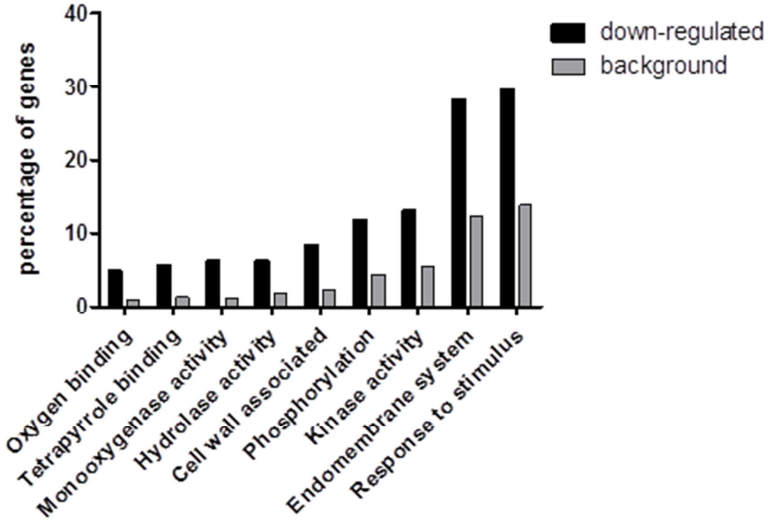


Figure 4.3: GO terms significantly enriched among up-regulated and down-regulated genes in *35S:ANAC032* plants

The GO term enrichment analysis was performed using the AgriGO tool and the 347 up-regulated (A) and 147 down-regulated (B) genes as input respectively. The background shown is the percentage of genes, belonging to different categories, present on the ATH1 Affymetrix array chip.

The up-regulated genes were also compared to genes identified in a previous array performed with RNA from mock and TIBA-induced wild-type *Col-0* plants (Ph.D. Thesis by Dr. Julia Köster). A total of seventy-eight genes were induced (>2-fold) in both arrays (Figure 4.4; list of the genes is mentioned in Supplementary Table S3). These genes could be potential downstream targets of ANAC032 in the detoxification response triggered by TIBA. Interestingly, some of these common genes were also the genes that depended on the TGA/SCL14 complex but did not contain an *as-1-like* element in their promoters (based on the SCL14 array mentioned above, (Fode et al., 2008)). Three of the common genes – the two highest up-regulated At2g37770 (*AKR4C9*) and At1g10585 (*bHLH585*) as well as At3g04000 [which is speculated to detoxify reactive carbonyl compounds; (Yamauchi et al., 2011)] were selected for further analysis. A fourth gene, *FLC* (*FLOWERING LOCUS C* (At5g10140)), a well-known transcriptional repressor/transcription factor regulating the initiation of flowering, was also selected for analysis.

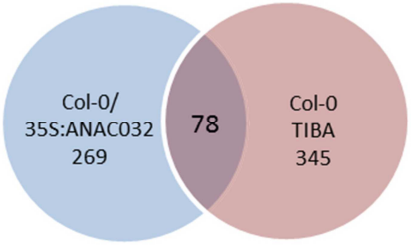


Figure 4.4: Venn diagram illustrating number of genes commonly up-regulated by TIBA and ectopic expression of ANAC032

The microarray results were first confirmed by qRT-PCR for the four selected genes which showed several fold up-regulations in plants overexpressing ANAC032. Additionally, it was observed that these genes are also induced when the other NAC TF *ATAF1* was over-expressed (Figure 4.5) suggesting redundancy between the two TFs in regulating induction of these genes.

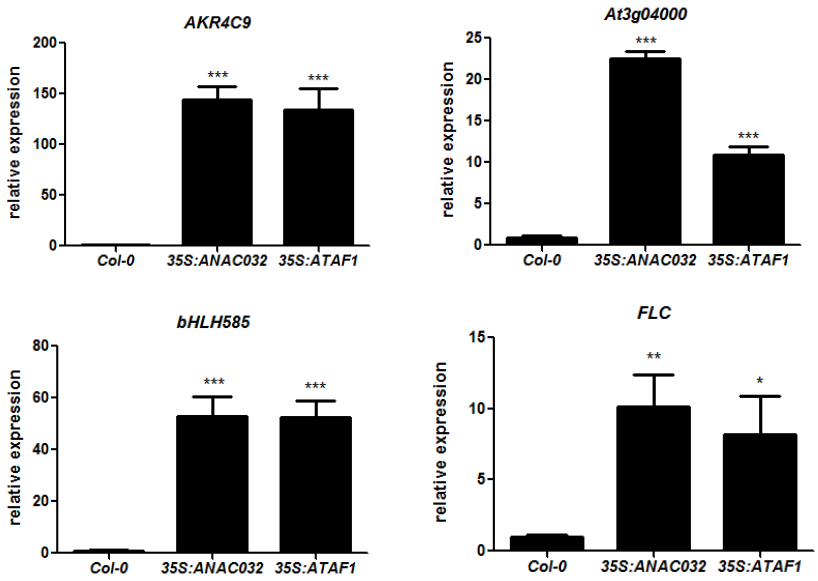


Figure 4.5: *AKR4C9*, *bHLH585*, *At3g04000* and *FLC* are up-regulated upon ectopic expression of *ANAC032* or *ATAF1*

Four-week old soil-grown (long day) *Col-0*, *35S:ANAC032* and *35S:ATAF1* plants were analyzed. The relative transcript levels (normalized to house-keeping gene *UBQ5*) of *AKR4C9*, *bHLH585*, *At3g04000* and *FLC* (indicated above each graph) were determined by qRT-PCR. The average relative expression value in *Col-0* was set to 1. The mean values (± SE) from five individual plants are shown. Asterisks indicate significant differences compared with wild-type (Two-way ANOVA; *** P<0.001, ** P<0.01, * P<0.05)

Next, single knockouts *ataf1* and *anac032* and the double knockout *ataf1anac032* were tested for compromised induction of *AKR4C9*, *bHLH585*, *At3g04000 and FLC* after application of TIBA. Three of the selected genes namely, *AKR4C9*, *bHLH585* and *At3g04000* were induced in *Col-0* upon TIBA application. This induction seemed to be compromised in the single knockouts as well as in the double knockout albeit in varying degrees for the three different genes. The induction of *AKR4C9* in the *ataf1* knockout was only slightly less as compared to wild-type whereas *anac032* and the double knockout *ataf1anac032* showed a strong suppression suggesting that *ANAC032* contributes more to the expression of *AKR4C9* than *ATAF1*. In the case of *bHLH585* and *At3g04000*, both single knockouts and the double knockout showed similar levels of compromised induction (Figure 4.6 A). Furthermore, the induction of

the three selected candidate genes was suppressed in the *tga256* and *scl14/33* knockout mutants suggesting that these are indeed indirect target genes in the detoxification pathway (Supplementary Figure S2).

The role of *AKR4C9*, *bHLH585* and *At3g04000* as direct targets of the NAC proteins is also supported by transient expression assays that were carried out in *ataf1anac032* leaf protoplasts. To this aim, the promoters of the target genes were fused to a luciferase reporter gene, while *ANAC032* and *ATAF1* were fused to the ubiquitin (*UBI10*) promoter. All three promoter constructs were activated by the NAC proteins when fused to the strong activating domain VP16 (Figure 4.6 B). Since the unfused NAC proteins were unable to activate the promoter constructs (Supplementary Figure S4), we suggest that the NAC proteins can bind to the target promoters but require additional factor(s) for transcriptional activation. All these data taken together suggest that *AKR4C9*, *bHLH585* and *At3g04000* are direct targets of ANAC032 and ATAF1 in the detoxification pathway triggered by the TGA/SCL14 complex. Nevertheless, in the case of all three candidate target genes, their induction by TIBA was not completely abolished in *ataf1anac032,* suggesting that perhaps other redundant NAC proteins (possibly ATAF2 and ANAC102) are at play.

The fourth gene selected for analysis – *FLC* – was not induced by TIBA and its promoter could not be significantly induced in transient protoplast expression assays suggesting that this gene may not be a direct target of the NAC TFs and may not play a role in the detoxification pathway (Figure 4.6). However, NAC-activated *FLC* expression might play a role in seed dormancy (see below).

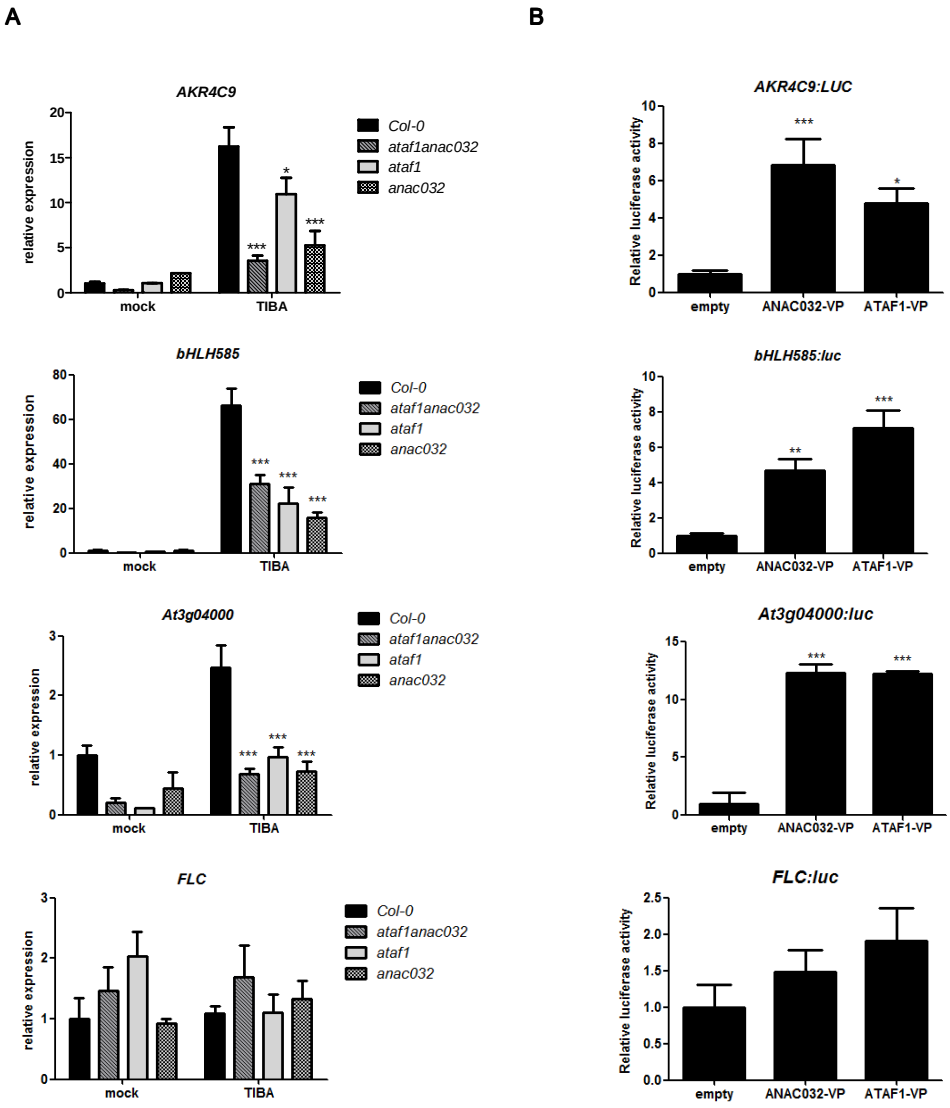


Figure 4.6: *AKR4C9*, *bHLH585* and *At3g04000* are possible direct targets of the ANAC032 and ATAF1 TFs in the detoxification response

(A) Six-week old soil-grown (short day) plants were treated with 0.1mM TIBA or with 0.1% DMSO (control) for 8 hours. The relative transcript levels (normalized to house-keeping gene UBQ5) of *AKR4C9, bHLH585, At3g04000* and *FLC* were determined by qRT-PCR. Average relative expression value in mock treated *Col-0* plants was set to 1. The mean values (±SE) from three individual experiments, each conducted with 5-6 individual plants/genotype are shown. Asterisks indicate significant differences compared with mock *Col-0* plants (One-way ANOVA; *** P<0.001, * P<0.05)

(B) Leaves from four-week old soil-grown *ataf1anac032* plants grown under 12-h light/12-h dark photoperiod were used for protoplast isolation and transformation with different effector and reporter constructs as indicated in the graphs. The values shown are means of two independent experiments, each with 5-6 reactions per effector construct. Asterisks indicate significant differences compared with transformation with empty vector (One-way ANOVA; *** P<0.001, **P<0.01, * P<0.05)

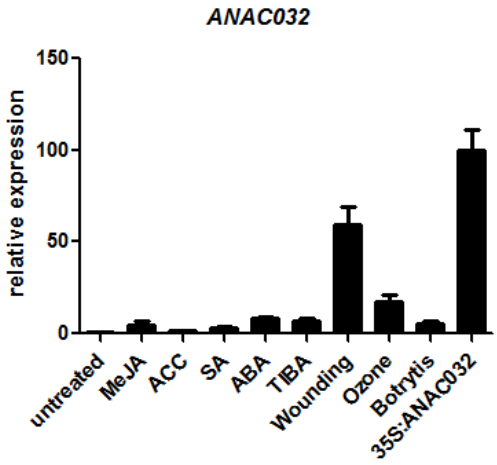
4.3 *ANAC032* and *ATAF1* and their downstream targets are involved in diverse stress responses

The ATAF subfamily members are predicted to be stress-responsive (Ooka et al., 2003). Genevestigator data show that they can be induced under variety of abiotic stress conditions, by application of most major hormones and also by pathogen infection (Figure 4.7 A). To better understand the induction pattern of *ANAC032* upon different stimuli and to find conditions where expression of the gene can reach levels found in the *35S:ANAC032* transgenic lines, wild-type plants grown together under same conditions were treated in different ways as indicated in Figure 4.7 B and C. Application with ABA (abscisic acid), MeJA (methyl jasmonate) and TIBA was analyzed 8 hours after treatment while ACC (1-aminocyclopropane-1-carboxylic acid; precursor of ethylene) and SA (salicylic acid) treatments were done for 24 hours. These time-points were selected because high induction of marker genes of the respective pathways can be observed for the different treatments. Under our conditions, *ANAC032* was not highly induced after application of the hormones MeJA, ACC or SA. It was rather significantly induced after treatments with ABA (abscisic acid), TIBA and after infection with *Botrytis cinerea* (roughly a 15-fold induction as compared to untreated plants). The highest induction was observed after wounding (60-80-fold) and also to some extent after ozone application (20-30-fold). *ANAC032* induction after wounding was on an average 60% of that found in the over-expressing plants (Figure 4.7 B). The *ATAF1* expression pattern was quite similar to *ANAC032* in that it showed no or very low induction after MeJA, ACC or SA at the time-points checked. There was a ~10-fold induction detected after ABA, TIBA or ozone treatments and ~20-fold induction after wounding and *Botrytis* infection. Even though wounding elicited the highest expression in this case, it was a mere 20% of that found in *35S:ATAF1* plants (Figure 4.7 C). Therefore, the effects observed in plants over-expressing *ATAF1* may be much stronger than ever achieved under natural conditions.

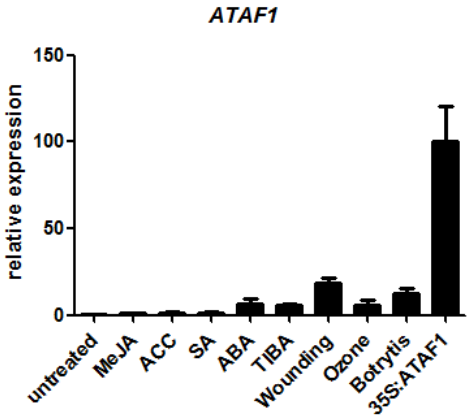
A


a-9 (ATAF TFs)
a-6
a-5
a-4
a-2
a-1
AT1G77450
AT1G01720
AT5G03790
AT1G69490
AT3G04070
AT1G01110
AT3G15510
AT1G52890
AT5G64530
AT1G52890
AT3G15500
AT4G27410
AT3G60560.
AT3G60530.
2,4,6 T / mock treated seedlings
2,4-dichlorophenoxyacetic acid / mock treated plant samples
4-thiazolidinone/acetic acid / solvent treated seedlings
AgNO3 / mock treated seedlings
CMP (24h) / solvent treated root culture samples (24h)
CMP (4h) / solvent treated root culture samples (4h)
cycloheximide / mock treated seedlings
DFPM (Col-0) / solvent treated seedling samples (Col-0)
dicamba (10h) / H2O treated seedling samples (10h)
fenclorim (24h) / solvent treated root culture samples (24h)
fenclorim (4h) / solvent treated root culture samples (4h)
furyl acrylate ester / solvent treated seedlings
hydrogen peroxide (Col-0) / untreated seedlings (Col-0)
ozone / air treated seedlings
phytoprostane A1 (cell culture) / solvent treated cell culture samples
phytoprostane A1 (Col-0) / solvent treated (Col-0) seedlings
TIBA / mock treated seedlings
B. cinerea / non-infected rosette leaf samples
B. graminis (Col-0) / non-infected rosette leaf samples
P. syringae pv. maculicola (Col-0) / mock treated leaf samples (Col-0)
P. syringae pv. tomato study 12 (Col-0) / untreated leaf tissue samples (Col-0)
ABA (1h) / mock treated seedlings (1h)
ABA (30min) / mock treated seedlings (30min)
ABA (3h) / mock treated seedlings (3h)
ABA study 4 (Col-0) / mock treated leaf samples (Col-0)
ABA study 5 (Col-0) / untreated plant samples (Col-0)
ABA study 6 (Col-0) / untreated plant samples (Col-0)
MeJa study 2 (3h) / mock treated seedlings (3h)
MeJa study 3 (0.5h) / solvent treated cell culture samples (0.5h)
MeJa study 3 (2h) / solvent treated cell culture samples (2h)
MeJa study 3 (6h) / solvent treated cell culture samples (6h)
MeJa study 4 (Col-0) / mock treated plant samples (Col-0)
salicylic acid / mock treated seedlings
salicylic acid study 10 (4d) / mock treated Col-0 rosette leaf samples (4d)
high light (Col-0) / low light grown seedlings (Col-0)
iron deficiency / protoplasting / iron deficiency study 8 (24h)
shift to pH 4.6 / protoplasting / shift to pH 4.6 (24h)
drought study 16 (Col) / mock treated Col whole plant samples
drought study 6 (Col-0) / untreated plant samples (Col-0)
hypoxia study 14 (Col-0) / mock treated rosette samples (Col-0)
osmotic study 4 (root) / mock treated Col-0 root samples
osmotic study 4 (shoot) / mock treated Col-0 shoot samples
salt study 3 (Col-0) / H2O treated rosette leaf samples (Col-0)
wounding (early) / untreated green tissue samples (early)
wounding (late) / untreated green tissue samples (late)

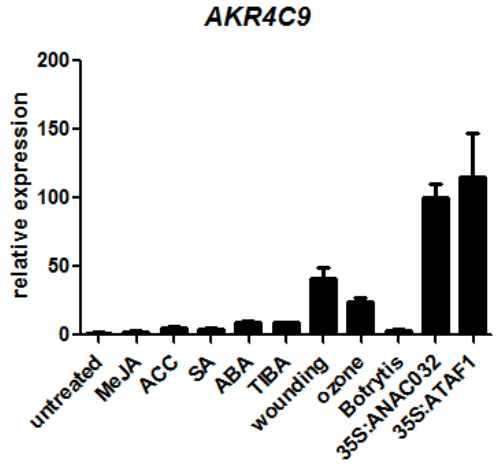
B


ANAC032
relative expression
150
100
50
0
untreated
MeJA
ACC
SA
ABA
TIBA
Wounding
Ozone
Botrytis
35S:ANAC032

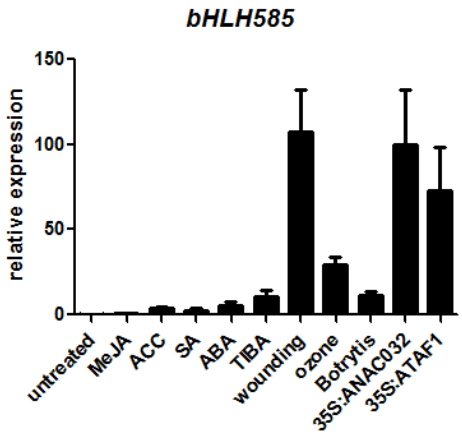
C


ATAF1
relative expression
150
100
50
0
untreated
MeJA
ACC
SA
ABA
TIBA
Wounding
Ozone
Botrytis
35S:ATAF1

D


AKR4C9
relative expression
200
150
100
50
0
untreated
MeJA
ACC
SA
ABA
TIBA
wounding
ozone
Botrytis
35S:ANAC032
35S:ATAF1

E



F

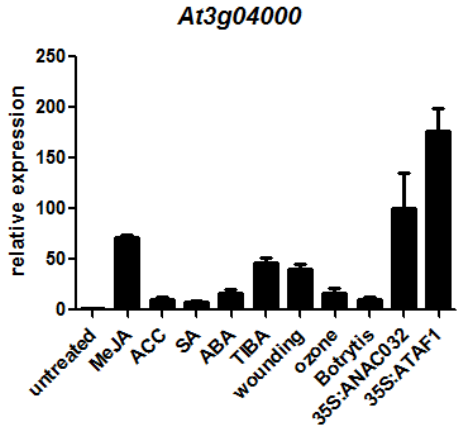


Figure 4.7: ***ANAC032* and *ATAF1* and their target genes can be significantly induced under various stress conditions**

A: Comparison of the transcript profiles of *A.thaliana* NAC TFs belonging to NAC-a-9 motif clade (ATAF proteins; within the yellow box) and other closely-related members of NAC-a sub-family (motif clades indicated above) in response to various stimuli. Data were extracted from Genevestigator microarray database (Hruz et al., 2008).

B-F: Four-week old soil-grown *Col-0* plants were used for different treatments. MeJA application was achieved via gaseous phase treatment of plants for 8hours. Plants were treated with 1mM ACC or 1mM SA for 24 hours and with 0.1mM ABA or 0.1mM TIBA for 8 hours by spraying the chemicals on the leaf surface. Plants were treated with 0.3ppm ozone for 6 hours. Wounding was achieved by use of forceps and samples were harvested 90 min later. Infection by *Botrytis cinerea* was performed by spot inoculating 6µl of 50,000 spores/ml culture onto the leaf surface and infected leaves were harvested 3dpi. Untreated plants served as control. The relative transcript levels (normalized to house-keeping gene UBQ5) of *ANAC032* (B), *ATAF1* (C), *AKR4C9* (D), *bHLH585* (E) or *At3g04000* (F) in untreated and treated wild-type plants is compared to untreated *35S:ANAC032* (B, D, E and F) or untreated *35S:ATAF1* (C) plants which is set to 100.

The target genes identified after TIBA application were also analyzed for their expression under these different conditions. As shown in Figure 4.7 C and D, *AKR4C9* and *bHLH585* followed a pattern very similar to *ANAC032*. Both genes were induced slightly after ABA and TIBA application (~10-fold induction), moderately high after ozone treatment (~25-fold induction) and showed maximum induction after wounding (~50-fold for *AKR4C9* and ~100-fold for *bHLH585*). Unlike *AKR4C9*, the *bHLH585* was also significantly induced after *Botrytis* infection (~12-fold). The third gene, *At3g04000*, showed a slightly different pattern (Figure 4.7 E) in that it was induced after all treatments and showed maximum induction after MeJA (~60-fold induction) while its induction after wounding was little bit lower (~40-fold) in the same range as after induction with TIBA. It was also induced after ABA and ozone treatment (~15 fold) and also slightly after ACC, SA and *Botrytis* infection (~8-fold). These expression profiles suggest that these genes are not specific to the detoxification triggered by a xenobiotic compound like TIBA. Instead they may be general stress-responsive enzymes (*AKR4C9* and *At3g04000*) or transcription factors (*bHLH585*) that are triggered under different conditions by the NAC proteins for detoxification of stress-induced toxic compounds/metabolites.

The induction of *ANAC032* after TIBA requires class-II TGA factors and SCL14 (Figure 4.1 B). Similarly, its induction and also the induction of the three target genes, requires the TGA and SCL14 factors after wounding (Figure 4.8 A and B). However, not all three genes showed compromised induction in wounded *ataf1anac032* plants (Figure 4.8 C) The *AKR4C9* had significantly lower expression in wounded knockout plants. Expression of *At3g04000* after wounding was less significantly affected while *bHLH585* expression was unaffected in the double knockout. This could again be an indication that TGA/SCL14 triggers expression of other ATAF TFs which contribute to the expression of these target genes. Indeed as shown in Figure 4.8 D, *ANAC102* and *ATAF2* are both significantly induced upon wounding.

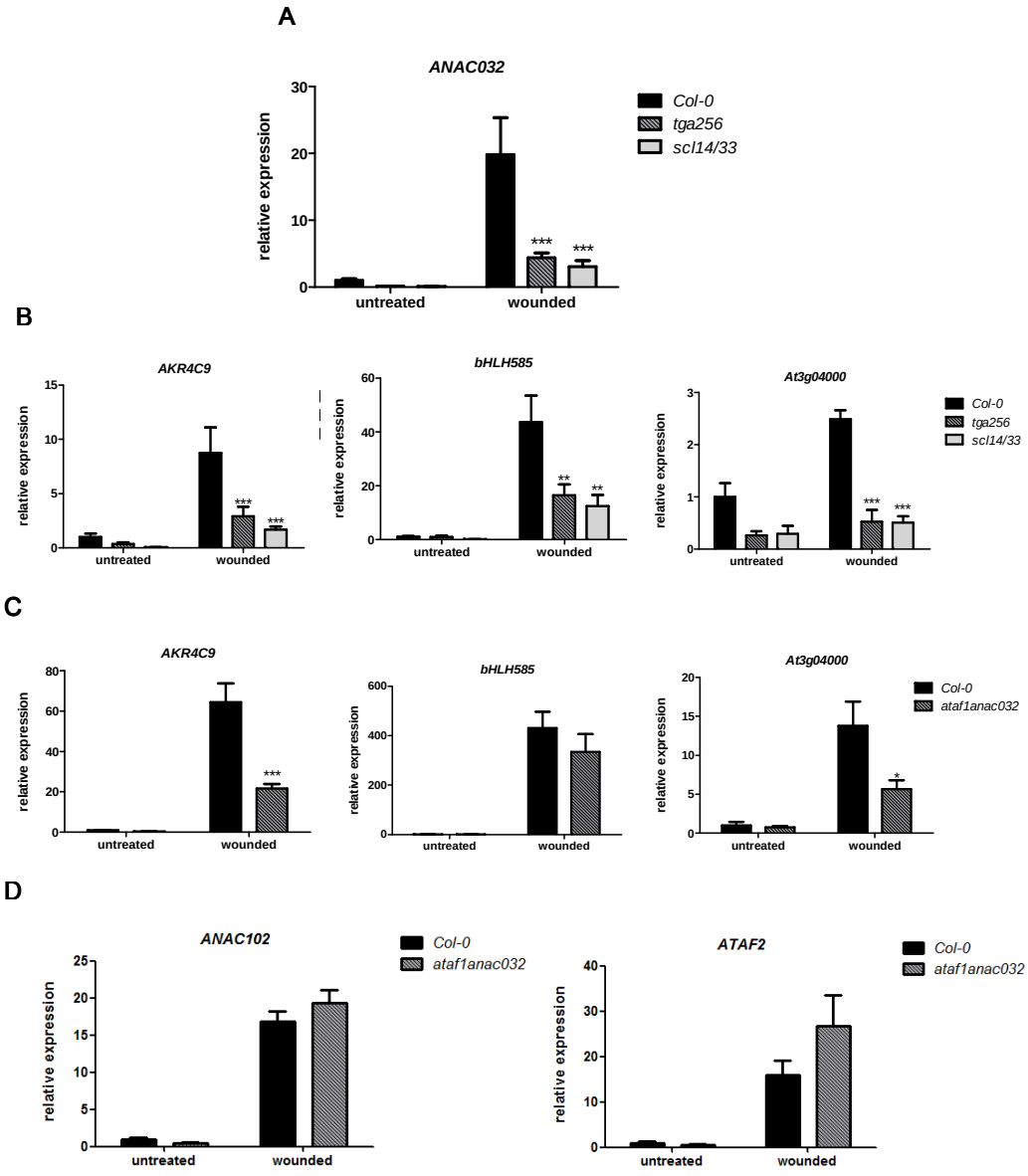


Figure 4.8: Wound-induced *ANAC032* and its target genes also require TGA and SCL14 factors

Four-week old soil-grown (long day) plants were wounded using a forceps and material was harvested 90 minutes later. Untreated plants served as control. The relative transcript levels (normalized to house-keeping gene UBQ5) of (A) *ANAC032* or (B and C) *AKR4C9*, *bHLH585* and *At3g04000* or (D) *ANAC102* and *ATAF2* was determined by qRT-PCR. The average relative expression value in untreated *Col-0* plants was set to 1. The mean values (±SE) from 5-6 individual plants are shown. Asterisks indicate significant differences compared with wild-type (Two-way ANOVA; *** P < 0.001; ** P<0.01)

4.4 *ANAC032* plays a JA-independent role in wound response

To understand further the probable role of *ANAC032* in the wound response, plants over-expressing *ANAC032* and knockout mutant plants were wounded and gene expression of the well-known wound-responsive gene *VSP2* was analyzed (Figure 4.9). Both *35S:ANAC032* and *35S:ATAF1* showed compromised induction of *VSP2* after mechanical wounding indicating a negative role of these NAC TFs. No difference was observed between *Col-0* and *ataf1anac032* (Figure 4.9).

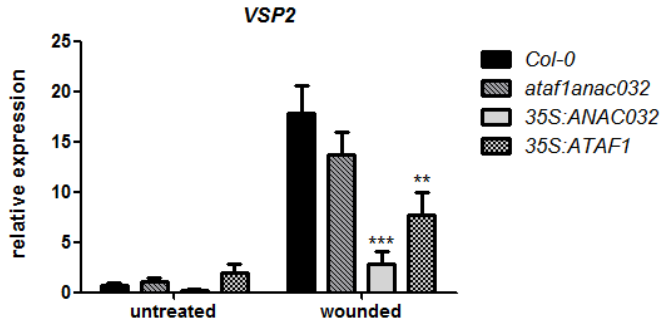


Figure 4.9: *ANAC032*- and *ATAF1*-overexpression suppresses *VSP2* induction after wounding

Four-week old soil-grown (long day) plants were wounded using a forceps and material was harvested 90 minutes later. Untreated plants served as control. The relative transcript levels (normalized to house-keeping gene UBQ5) of *VSP2* was determined by qRT-PCR. The average relative expression value in untreated *Col-0* plants was set to 1. The mean values (± SE) from three individual experiments, each conducted with 5-6 individual plants are shown. Asterisks indicate significant differences compared with wild-type (Two-way ANOVA; *** $P < 0.001$; ** $P<0.01$)

VSP2 expression in *Arabidopsis* is a jasmonic acid (JA)-mediated response (Berger et al., 1996; Staswick et al., 1992). In order to elucidate whether the induction of *ANAC032* after wounding was also JA-mediated, wounding experiments were carried out with the JA-biosynthesis mutant *dde2-2* (knockout mutation in *ALLENE OXIDE*

SYNTHASE (*AOS*), (Park et al., 2002a)) and JA-receptor mutant *coi1-t* (T-DNA insertion within *COI1* locus, (Mosblech et al., 2011)). As shown in Figure 4.10, wounding highly induced *ANAC032* in wild-type, but it was also induced in both knockout mutants suggesting that JA is not involved in wound-induced *ANAC032* expression.

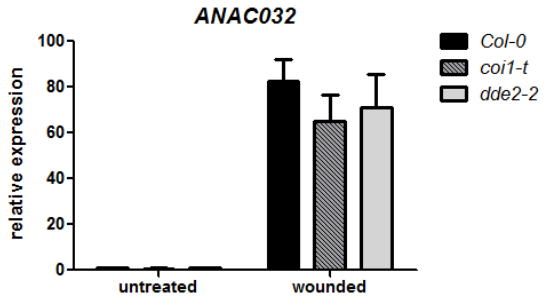


Figure 4.10: Wound-induced *ANAC032* expression is JA-independent

Four-week old soil-grown (12h light/12h dark photoperiod) plants were wounded using a forceps and material was harvested 90 minutes later. Untreated plants served as control. The relative transcript levels (normalized to house-keeping gene *UBQ5*) of *ANAC032* was determined by qRT-PCR. The average relative expression value in untreated *Col-0* plants was set to 1. The mean values (±SE) from two experiments each with six individual plants are shown. Analysis by Two-way ANOVA suggests no significant differences between the genotypes.

In contrast to the JA-dependent wound response, which leads to systemic induction of gene expression, the JA-independent response which can be induced by oligogalacturonides, was shown to be restricted to the local wound site ((Rojo et al., 1999); See section 1.3). Therefore, local wounded leaves and systemic unwounded leaves were harvested separately and analyzed for *ANAC032* gene expression. Indeed, as shown in Figure 4.11 A, *ANAC032* was induced only in local tissues after wounding. Furthermore, the induction seemed to be transient, the expression peaked within 90 min and then gradually decreased with induction reducing to less than half within 6 hours (Figure 4.11 B). Other than mechanical wounding, a common source

of injury is herbivory. Although there are common genes induced by both challenges, there are also genes induced specifically for either of the stresses (Reymond et al., 2000). Moreover, herbivory provides a situation where continuous wounding occurs due to feeding by larvae and this might lead to a prolonged expression of *ANAC032*, thus closely resembling the state in *35S:ANAC032* plants. To assess the behavior of *ANAC032* under such a situation, wild-type and *ataf1anac032* mutant plants were challenged with the specialist herbivore *Plutella xylostella* and the feeding pattern was observed for two days until when the larvae had fed on approximately half the plant tissue. The remaining tissue was harvested and analyzed for gene expression. Firstly, as shown in Figure 4.11 C, the specialist performed equally well on both genotypes as indicated by their body weight. Secondly, *ANAC032* was also induced upon insect challenge (Figure 4.11 D) although to a low degree (~10 fold) as compared to its induction after mechanical wounding (which can reach up to 60-80 folds).

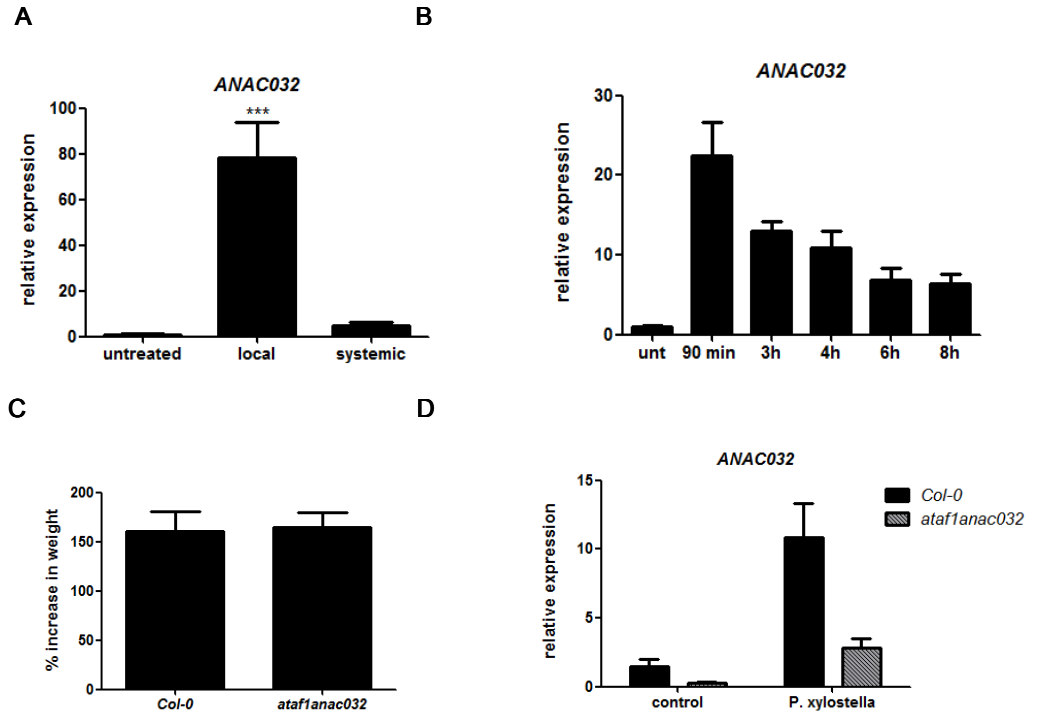


Figure 4.11: *ANAC032* is locally and transiently induced upon wounding of tissue

A and B: Three week old soil-grown (long day) *Col-0* plants were wounded using a forceps such that almost the entire leaf area was injured. Local wounded leaves and systemic unwounded leaves from wounded plants were harvested separately after 90 min (A) or local wounded leaves after time points 90 min, 3h, 4h, 6h and 8h were collected (B). Untreated plants served as control. The relative transcript levels (normalized to house-keeping gene UBQ5) of *ANAC032* was determined by qRT-PCR. The average relative expression value in untreated *Col-0* plants was set to 1. The mean values (±SE) from two experiments each with 5 or more individual plants are shown. Asterisks indicate significant differences compared with wild-type (Two-way ANOVA; *** $P < 0.001$; ** $P<0.01$).

C and D: First instar of specialist herbivore *Plutella xylostella* were placed on three week old (long-day) *Col-0* and *ataf1anac032* plants and half-eaten leaves were harvested 2 days later. Untreated plants served as control. The larvae weight gain (shown in percentage) was measured two days after feeding (C). The relative transcript levels (normalized to house-keeping gene UBQ5) of *ANAC032* was determined by qRT-PCR. The average relative expression value in untreated *Col-0* plants was set to 1 (D). The mean values (±SE) are from ten individual plants or insects.

4.5 Over-expression of *ANAC032* suppresses JA-, ET- and SA- induced gene expression

Wound-induced *VSP2* expression was reduced in plants ectopically expressing the NAC TFs. To find out, whether this is due to interference with the JA signaling pathway, the effect of the TFs on *VSP2* expression was studied after external application of MeJA. As observed after wounding, the induction was suppressed in the *35S:ANAC032* plants (Figure 4.12 A). Since JA in the presence of ET leads to induction of defense genes like *PDF1*.2, the effect of the NAC TFs on this branch of the JA signaling network was examined by treating plants with ACC, the precursor of ET. *PDF1.2* induction was also suppressed in the *35S:ANAC032* plants while no difference could be observed between wild-type and *ataf1anac032* knockout plants (Figure 4.12 C). In both kinds of treatment the *35S:ATAF1* plants behaved like the *35S:ANAC032* plants. Further, it was observed that the suppression took place at the level of transcription of *MYC2* and *ORA59*, which are upstream regulatory factors in JA and JA/ET pathways respectively (Figure 4.12 B and D).

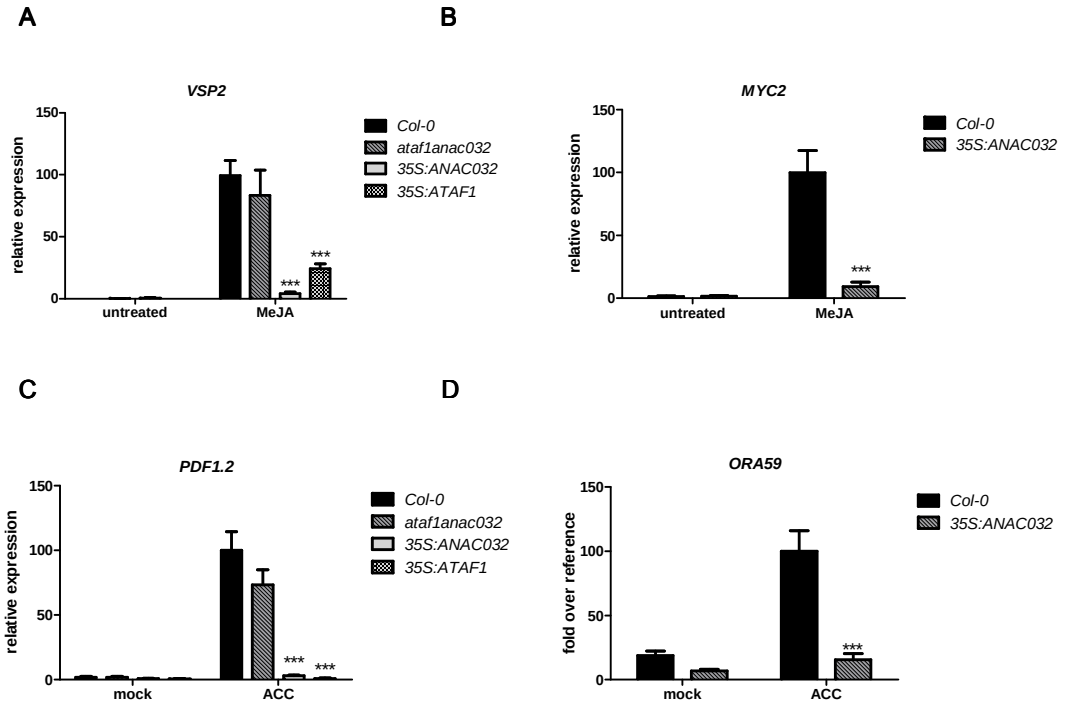


Figure 4.12: Over-expression of *ANAC032* suppresses induction of JA and JA/ET-responsive genes

Four week old soil-grown (long day) plants were treated with 4.5µM MeJA via the gaseous phase for 8 hours (A) or with 1mM ACC for 24 hours (B). Untreated (A) or water treated (B) plants served as controls. The relative transcript levels (normalized to house-keeping gene UBQ5) of *VSP2* (A), *MYC2* (B), *PDF1.2* (C) or *ORA59* (D) was determined by qRT-PCR. The average relative expression value in treated *Col-0* plants was set to 100. The mean values (± SE) from three experiments each with 5 or more individual plants are shown. Asterisks indicate significant differences compared with wild-type (Two-way ANOVA; *** $P < 0.001$).

Salicylic acid (SA) is known to suppress the JA/ET pathway and this cross-talk depends on class-II TGA TFs (Ndamukong et al., 2007; Zander et al., 2012). Since SA can induce the NAC TFs in a class-II TGA-dependent manner (~2-fold, after 24h treatment), we speculated that part of this cross-talk is mediated by *ANAC032*, To address this, cross-talk experiments were carried out where *Col-0*, *ataf1anac032* and *35S:ANAC032* plants were treated either with ACC alone or with both ACC and SA

simultaneously. Twenty-four hours after treatment with ACC, both *Col-0* and *ataf1anac032* showed similar levels of induction of *PDF1.2* and as observed before the *35S:ANAC032* plants strongly suppressed this induction. After additional SA treatment the *Col-0* plants showed reduced *PDF1.2* induction due to antagonistic effect of the SA pathway. This suppression was however also observed in the *ataf1anac032* plants suggesting that NAC proteins might not play a role in the cross-talk (Figure 4.13 A). Still, the potential redundancy with the other two NACs and with maybe NAC-independent mechanisms has to be considered.

Interestingly, when *PR1* induction was analyzed to control for the efficiency of the SA treatment, *PR1* was also found to be suppressed in the *35S:ANAC032* plants treated with SA and ACC. This was then confirmed by a second experiment where plants were treated with SA alone and *PR1* expression was compromised in *35S:ANAC032* as well as in *35S:ATAF1* plants (Figure 4.13 B). The ABA hormone pathway is also known to have antagonistic effects on the defense pathways leading to *PR1* (SA-pathway) and *PDF1.2* (JA/ET pathway) suppression. Thus, a cross-talk experiment was also carried out where the plants were simultaneously treated with the hormones ABA and ACC (Fig 4.13 C). Here again *ataf1anac032* knockout mutant behaved like the wild-type and showed suppression of the *PDF1.2* gene when both hormones were applied together suggesting that *ANAC032* is not required for the ABA-mediated cross-talk. Additionally, the ABA-responsive gene *COR78* was suppressed by over-expression of *ANAC032* (Figure 4.13 D).

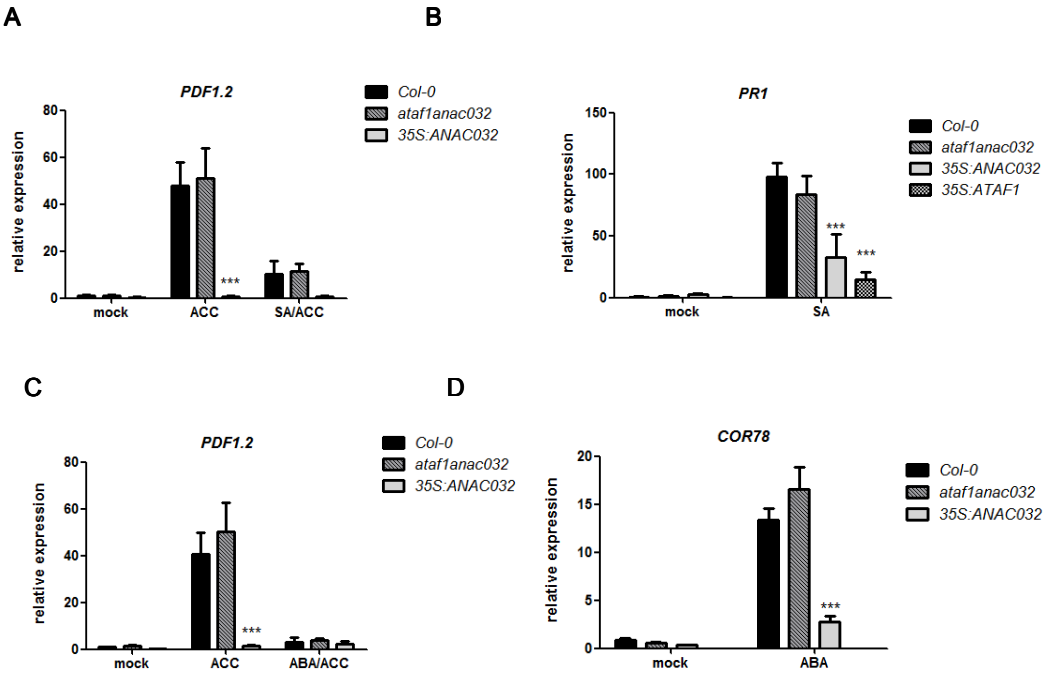


Figure 4.13: *ANAC032* may not play a role in different hormonal cross-talks

A and B: Four week old soil-grown (long day) plants were treated either with 1mM ACC, 1mM SA or simultaneously with both hormones for 24 hours. Water treated plants served as controls. The relative transcript levels (normalized to house-keeping gene UBQ5) of *PDF1.2* (A) or *PR1* (B) was determined by qRT-PCR. The average relative expression value in control *Col-0* plants was set to 1. The mean values (±SE) from 5 or more individual plants are shown. Asterisks indicate significant differences compared with wild-type (Two-way ANOVA; *** $P < 0.001$).

C and D: Four week old soil-grown (long day) plants were treated either with 1mM ACC, 100µM ABA or simultaneously with both hormones for 24 hours. Water treated plants served as controls. The relative transcript levels (normalized to house-keeping gene UBQ5) of *PDF1.2* (A) or *COR78* (B) was determined by qRT-PCR. The average relative expression value in control *Col-0* plants was set to 1. The mean values (±SE) from 5 or more individual plants are shown. Asterisks indicate significant differences compared with wild-type (Two-way ANOVA; *** $P < 0.001$).

It was observed previously that in protoplasts, the expression of *PDF1.2* and *VSP2* is strongly suppressed (Thesis by Julia Köster). Genevestigator data suggested that *ANAC032* and *ATAF1* gene expression is highly induced in response to protoplasting which could indeed be reproduced (Figure 4.14 A). Therefore it was speculated that this high induction of the NAC proteins during protoplast isolation might be mirroring the state in the over-expressing plants leading to the suppressed expression of the defense genes in protoplasts. However as shown in Figure 4.14 B, protoplasts prepared from wild-type and *ataf1anac032* plants showed similar levels of suppression of both genes.

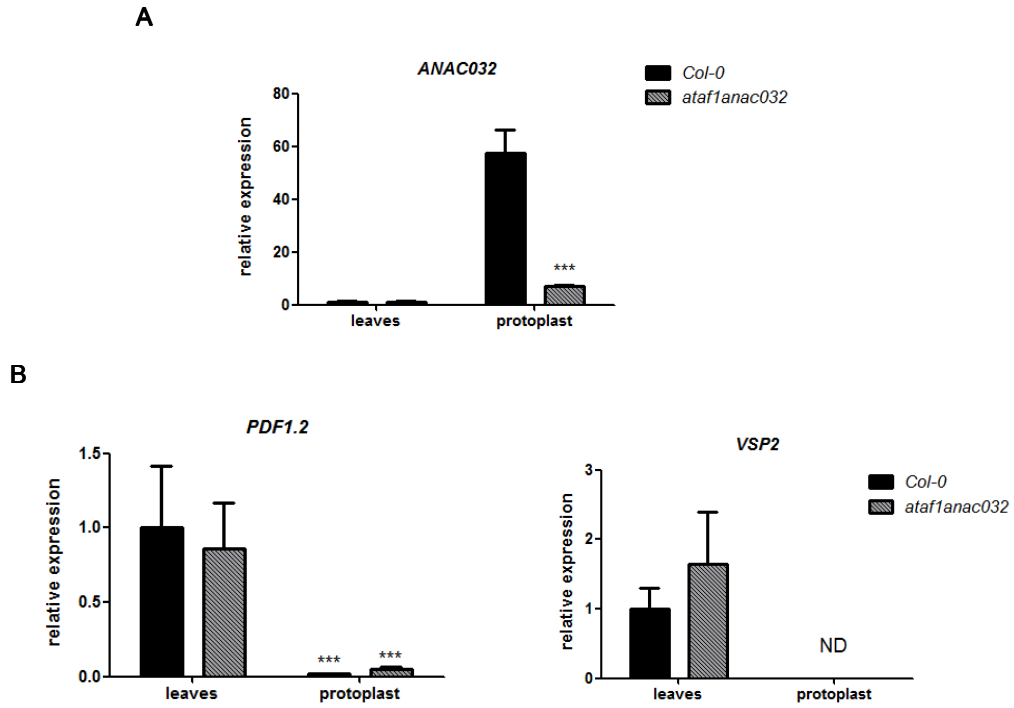


Figure 4.14: *ANAC032* does not contribute to suppression of *PDF1.2* and *VSP2* in protoplasts

Four week old plants were used to isolate protoplasts and as control leaves were also harvested. The relative transcript levels (normalized to house-keeping gene UBQ5) of *ANAC032* (A) or *PDF1.2* and *VSP2* (B) was determined by qRT-PCR. The average relative expression value in leaves of *Col-0* plants was set to 1. The mean values (±SE) from five individual plants are shown. Asterisks indicate significant differences compared with wild-type (Two-way ANOVA; *** $P < 0.001$). ND = not determined

As a first step towards understanding the mode of suppression of various signal cascades by NAC TFs, yeast two-hybrid studies were carried out. We tested whether ATAF subfamily proteins could interact with some known key players of the different pathways like EIN3 and TGA factors 2 and 5, which are important for the induction of *PDF1.2* upon ET treatment. Moreover, the potential interaction between NACs and GRX480, a negative regulator of the JA/ET pathway, was tested. However the NAC proteins did not interact with any of them (data not shown). All these results propose that overexpression of *ANAC032* suppresses JA-, JA/ET- , SA- and ABA-induced gene expression via a yet-unknown mechanism.

4.6 Over-expression of *ANAC032* most likely affects signaling and not biosynthesis of phytohormones

The suppression of hormone-responsive genes could be either due to defective hormone accumulation or altered signaling. Given that the NACs induce the detoxification program, it might be assumed that the applied hormones are inactivated. Therefore, basal and induced levels of different hormones and their related metabolites were measured in plants with elevated or reduced expression levels of ANAC032 and ATAF1. For this purpose we chose to treat the plants with ozone since this treatment can at the same time trigger the accumulation of all three defense phytohormones ET, JA and SA (Kangasjärvi et al., 1994; Rao et al., 2000, 2002). The outcome of two independent experiments is documented in Figure 4.15 and 4.16. First, SA levels were increased after ozone treatment to the same degree in *Col-0* and the *ataf1anac032* mutant. Basal SA levels were slightly elevated in *35S:ANAC032* and *35S:ATAF1* plants. In contrast, ozone-induced SA levels were lower than in the wild-type in the first experiment but not in the second (Figure 4.15 A and B respectively). Similar to tobacco, SA in *Arabidopsis* is thought to be stored in the vacuole as SAG (Dean et al., 2005; Dempsey et al., 2011). There was slight or no increase in the SAG levels upon ozone treatment in *Col-0* and *ataf1anac032* plants. In contrast, SAG levels were high in *35S:ANAC032* plants from beginning on in both experiments and in *35S:ATAF1* plants in one of the experiments (Figure 4.15

C and D). This correlates well with increased expression of *UGT74F2/SAGT1* in *35S:ANAC032* plants as indicated by the microarray (~19-fold induction).

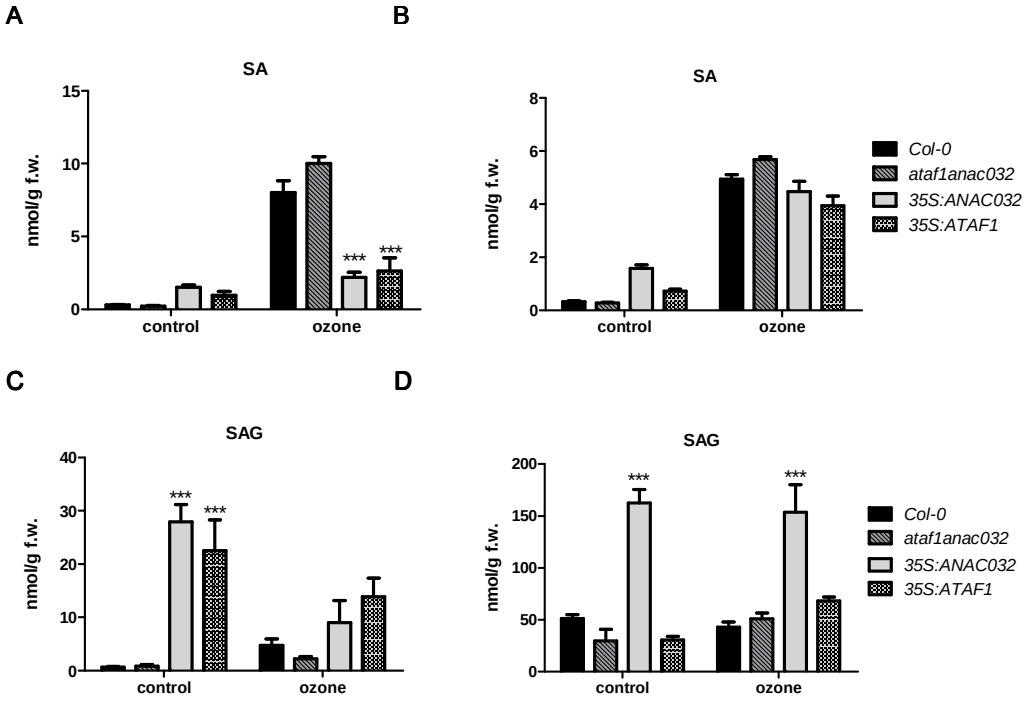


Figure 4.15: Accumulation of SA is not suppressed in *35S:ANAC032*

Four week old (long day) plants were treated with 0.3ppm ozone for 6 hours. Untreated plants served as control. The total SA (A and B) and SAG (C and D) contents were measured by HPLC MS/MS technique. The graphs A and C belong to the first experiment while B and D belong to the second experiment. The first experiment consisted of four individual plants for *Col-0* and *ataf1anac032* and four pools, each of four individual plants, for the over-expressing plants as biological replicates. The second experiment consisted of four pools of four individual plants for all genotypes as biological replicates. The mean values (±SE) of the replicates are shown. Asterisks indicate significant differences compared with wild-type (Two-way ANOVA; *** $P<0.001$).

JA levels were increased in all genotypes after ozone treatment and levels were again comparable between wild-type and *ataf1anac032* mutant plants. However, *35S:ANAC032* (but not *35S:ATAF1*) accumulated more JA after ozone treatment and in one of the experiments the basal levels were also much higher (Figure 4.16 A and B). The amount of the JA-precursor 12-Oxo-Phytodienoic Acid (OPDA) did not

significantly differ between genotypes and showed no increase after treatment in both experiments (figure 4.16 C and D). Thus, increased JA levels should be due to the increased activity of enzymes that convert OPDA to JA to JA-Ile. The microarray data indicates that *ACX1* (*ACYL-COA OXIDASE 1*) is induced (~2.5-fold) in the *35S:ANAC032* plants. This enzyme catalyzes the first β-oxidation step in the synthesis of JA from OPDA (Schilmiller et al., 2007) and thus may account for the observed increased levels of JA. The amino acid derivative JA-isoleucine (JA-Ileu) and degradation product hydroxyjasmonate (11, 12-OH-JA) show a similar behavior like JA with increased amounts observed in *35S:ANAC032* plants (Figure 4.16 E, F and G, H respectively). Therefore, it seems unlikely that the decreased transcript levels of *VSP2* and *PDF1.2* are due to the degradation of active JA.

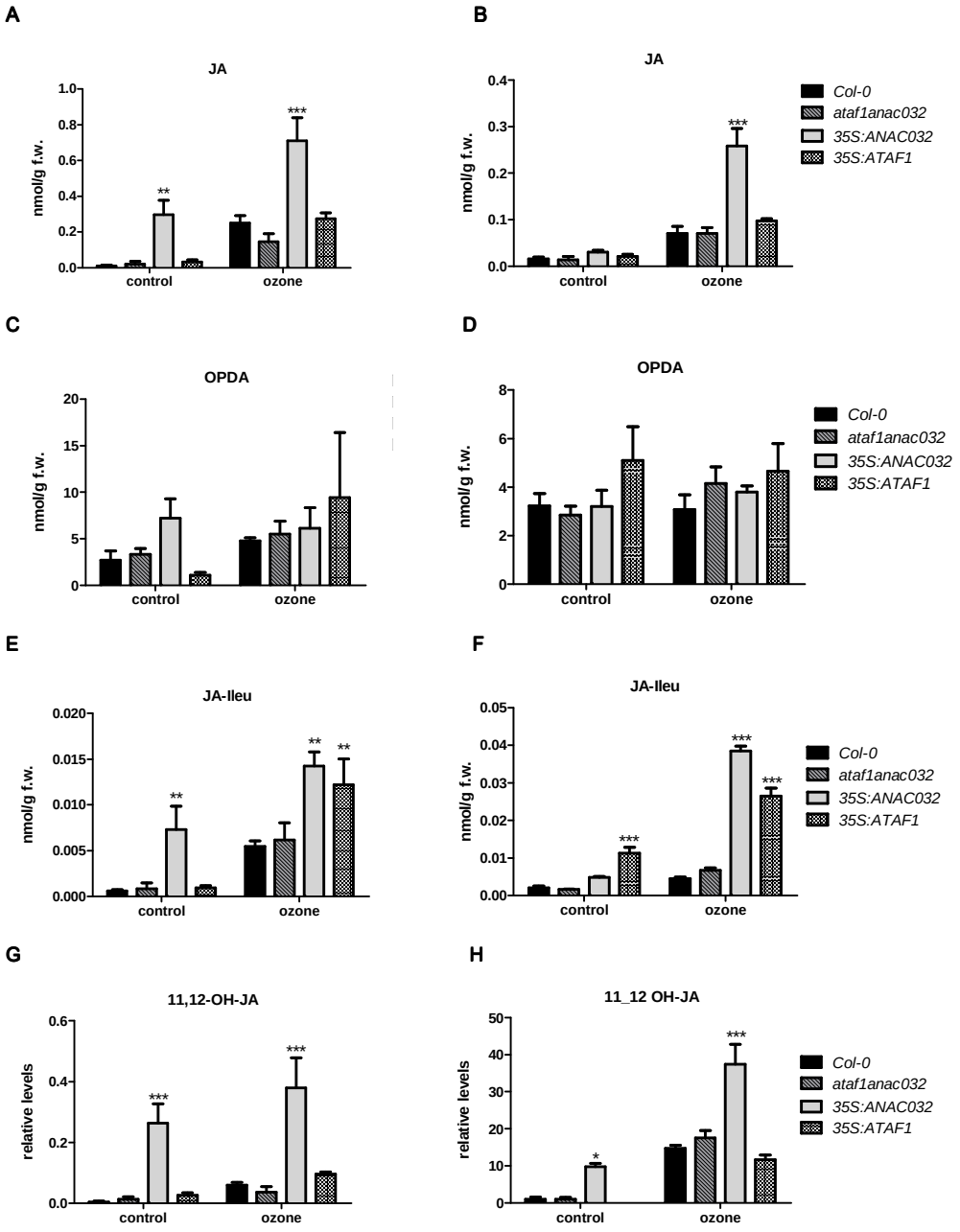


Figure 4.16: Accumulation of JA and JA-Ileu is not suppressed in *35S:ANAC032*

4-week old soil-grown (long day) plants were treated with 0.3ppm ozone for 6 hours. Untreated plants served as control. The total JA (A, B), OPDA (C, D), JA-Ileu (E, F) and 11, 12-OH-JA (G, H) contents were measured by HPLC MS/MS technique. The graphs A, C, E and G belong to the first experiment while B, D, F and H belong to the second experiment. The first experiment consisted of four individual plants for *Col-0* and *ataf1anac032* and four pools, each of four individual plants, for the over-expressing plants as biological replicates. The second experiment consisted of four pools of four individual plants for all genotypes as biological replicates. The mean values (±SE) of the replicates are shown. Asterisks indicate significant differences compared with wild-type (Two-way ANOVA; *** P<0.001, **P<0.01 and *P<0.05)

4.7 Over-expression of *ATAF1* leads to increased basal levels of abscisic acid (ABA)

As shown above, over-expression of *ANAC032* led to reduced induction of the ABA-responsive *COR78* gene (Figure 4.15 D). However over-expression of *ANAC032* did not affect basal ABA levels. On the other hand, over-expression of *ATAF1* led to increased basal levels of ABA (Figure 4.17). Moreover, although ABA levels did not drastically change after ozone treatment in wild-type and *ataf1anac032*, both over-expressors showed more accumulation of the hormone (~4-fold and ~1.5-fold more in *35:ATAF1* and *35S:ANAC032* plants respectively). Conversely, the single knockouts, especially *ataf1*, did not accumulate any ABA after ozone treatment.

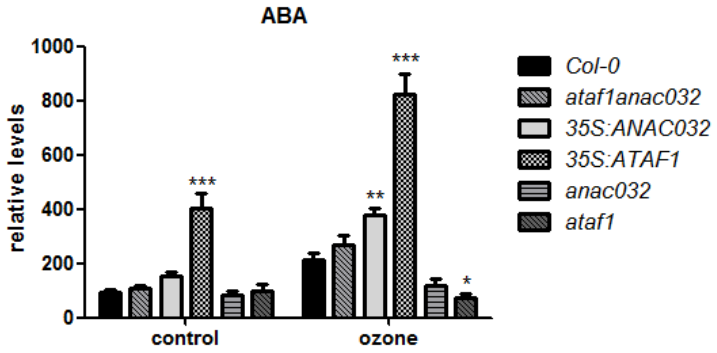


Figure 4.17: Over-expression of *ATAF1* leads to increased accumulation of ABA

Four week old long day grown plants were treated with 0.3ppm ozone for 6 hours. Untreated plants served as control. The ABA content was measured by HPLC MS/MS technique. Values (±SE) obtained from two independent ozone experiments are shown. The first experiment consisted of four individual plants for *Col-0* and *ataf1anac032* and four pools, each of four individual plants, for the over-expressing plants as replicates. The second experiment consisted of four pools of four individual plants for all genotypes as replicates. Asterisks indicate significant differences compared with wild-type (Two-way ANOVA; *** $P<0.001$, **$P<0.01$ and *$P<0.05$) (NOTE: Unlike, SA and JA levels shown above, the ABA levels were comparable between experiments and therefore single graph is plotted from all values obtained)

4.8 ANAC032 has a potential role in plant developmental processes

The *35S:ANAC032* and *35S:ATAF1* transgenic lines presented phenotypic differences compared to wild-type (Figure 4.18). The plants were dwarf in size, with crinkled and upward curled leaves that showed early yellowing along the veins and leaf edges (Figure 4.18 A). The size of the plant seemed to correlate with the amount of transgenic gene present. Homozygous plants of *35S:ANAC032* exhibited strong dwarfism, had an extended lifetime and were sterile producing only very short inflorescences. The heterozygous plants, on the other hand, displayed a less severe phenotype and were comparable to the wild-type in terms of size but still showed the early yellowing of the leaves. These plants were fertile and produced seeds so that the seed stock was maintained as a heterozygous pool with homozygous plants being selected for various experiments based on their small size. *35S:ANAC032* showed delayed flowering as shown in Figure 4.18 B. This might be due to the high expression of the floral repressor gene *FLC* in *35S:ANAC032* plants as indicated by the microarray analysis and qRT-PCR (Figure 4.5). Recently it was shown that *FLC* also plays a role in delaying the juvenile-to-adult vegetative transition and that it affects different leaf traits associated with vegetative phase change (Willmann and Poethig, 2011). Plants over-expressing *FLC* were shown to contain more number of juvenile or transition leaves which are characterized by more rounder leaves (lower length:width ratio) and by less number of abaxial trichomes compared to adult leaves. Consistent to this, the *35S:ANAC032* transgenic plants also have a higher number of juvenile leaves as judged by the number of abaxial trichomes present (Figure 4.17 C and D). Since *35S:ANAC032* plants are more juvenile it was checked whether in general juvenile leaves show reduced induction of defense genes. For this purpose, *JAZ10-GusPlus* reporter lines were treated with MeJA and GUS activity was quantified in juvenile and mature leaves separately. However both type of leaves showed no difference in their ability to respond to the hormone treatment (Figure 4.18 E).

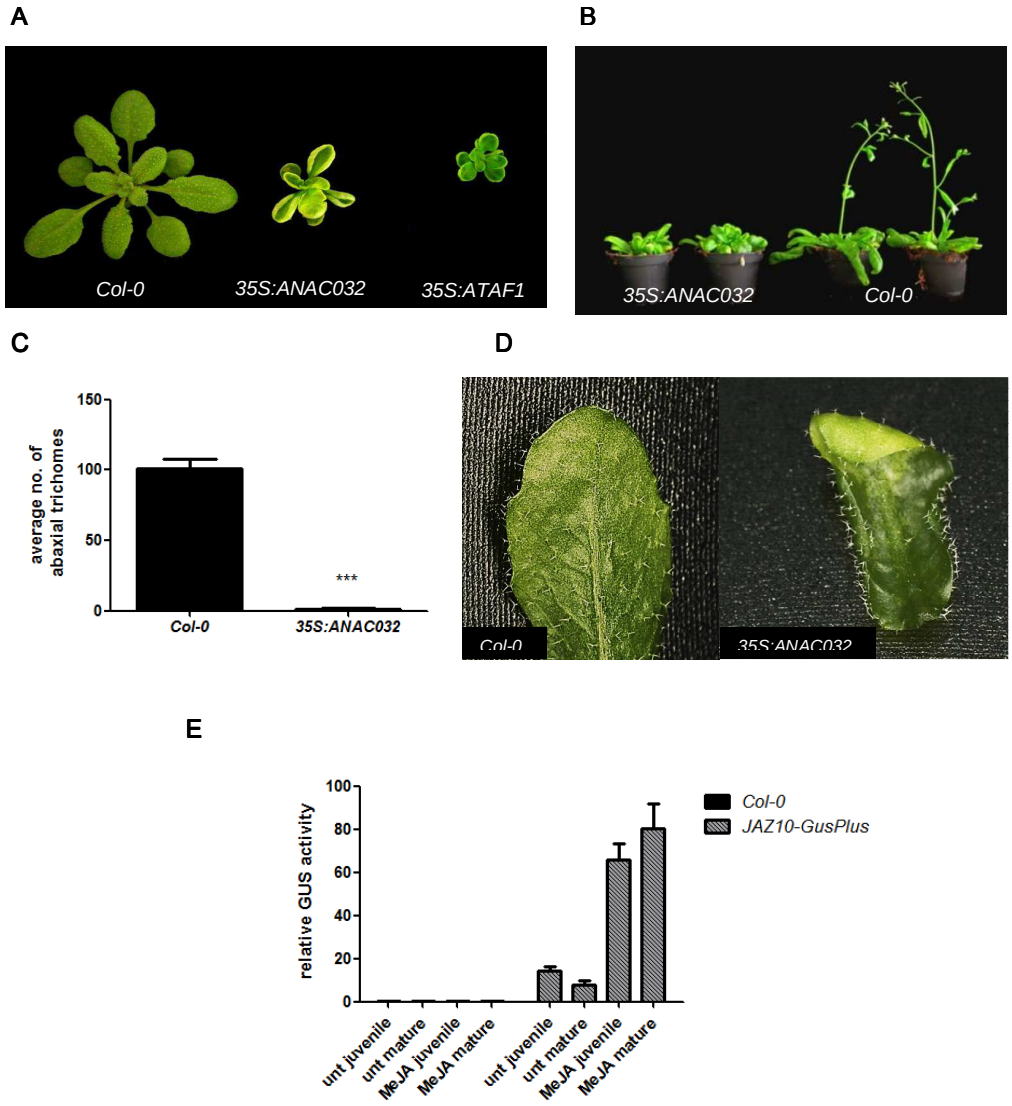


Figure 4.18: *35S:ANAC032* show altered phenotypic characteristics including increased juvenility

A. Plants were grown under long-day conditions and photographs taken 3-4 weeks later. **B.** 6-week old plants grown under long day conditions showing *Col-0* (right) plants already flowering while heterozygous *35S:ANAC032* (left) plants have not yet bolted. **C.** Average number of abaxial trichomes counted on the 8th to 10th leaf positions from ten plants. **D.** Representative photograph showing number of abaxial trichomes. **E.** Four week old long day grown plants were treated with 4.5µM MeJA via the gaseous phase for 8 hours. GUS activity was measured by MUG assay. *Col-0* plants and *JAZ10-GusPlus* untreated plants served as controls. The average relative GUS activities (±SE) from four individual plants are shown.

The transcriptomic database, Arabidopsis eFP browser [http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi; (Winter et al., 2007)] indicates that *ANAC032* and *ATAF1* transcript levels are very high in the seed stage (Figure 4.19). Moreover, the other members of the clade *ANAC102* and *ATAF2* do not show this high expression in seed and hence may not be functionally redundant in this context, providing a good chance to observe a phenotype for the *ataf1anac032* double knockout. Considering that *ANAC032* is a negative regulator of hormone signaling, we speculated that *ANAC032* might negatively regulate ABA-induced dormancy. Therefore, freshly harvested seeds *of Col-0* and *ataf1anac032* knockout mutants were placed on wet filter paper and observed for germination. Seeds that had been dried for over ten weeks (and hence fully released from dormancy) were used as controls. All seeds were first exposed to two days in the cold to partially break dormancy and then radicle emergence was scored three days later. As shown in Figure 4.20 A, C and E, there was no difference in the germination rate between completely dried *Col-0* and *ataf1anac032* seeds and 100% germination was observed for both within 3 days of incubation. Contrastingly, the freshly harvested seeds germinated more slowly where *Col-0* showed 80% germination and *ataf1anac032* showed only 18-20% germination (Figure 4.20 B, D and E). This supports our assumption that the NAC TFs are negative regulators of seed dormancy.

	ANAC032 (At1g77450)	ATAF1 (At1g01720)	ANAC102 (At5g63790)	ANAC029 (At1g69490)	ANAC047 (At3g04070)	ANAC025 (At1g61110)	ANAC056 (At3g15510)	ANAC018 (At1g52880)	ANAC104 (At5g64530)	ANAC019 (At1g52890)	ANAC055 (At3g15500)	ANAC072 (At4g27410)	ANAC065 (At3g56560)	ANAC064 (At3g56530)
Dry seed	1426.37	2227.75	727.81	15.16	6.34	1.2	4.07	9.59	4.9	205.58	10.58	819.3	8.52	4.52
Imbibed seed, 24 h	80.88	338.09	83.64	65.32	144.59	7.02	14.58	17.52	4.23	93.75	3.85	18.19	2.37	2.6
1st Node	17.61	51.61	94.06	16.05	19.81	1.83	12.68	64	33.53	17.64	2.18	34.8	1.53	3.4
Flower Stage 12, Stamens	74.91	330.61	116.68	30.36	49.86	1195.55	421.18	336.78	5.5	820.68	224.91	90.5	1.78	6.05
Cauline Leaf	99.93	236.15	331.36	1139.86	64.51	1.85	51.3	314.55	22.03	152.96	16.33	122.81	2.56	5.58
Cotyledon	39.33	66.91	179.93	24.73	28.85	2.48	86.65	155.01	12.53	20.95	29.08	347.48	4.45	4.98
Root	63.56	397.63	212.68	33.15	21.4	3.15	34.06	15.65	17.45	18.8	23.78	68.34	2.03	4.88
Entire Rosette After Transition to Flowering	17.03	53.41	110.11	85.38	19.83	1.56	26.2	77.43	10.46	23.2	11.68	78.96	4.64	4.5
Flower Stage 12, Carpels	18.23	348.36	136.88	7.06	14.28	5.2	4	25.2	13.35	119.3	6.65	19.98	3.06	3.4
Flower Stage 12, Petals	18.35	709.26	114.41	90.65	11.46	35.1	16.58	49.5	13.91	280.06	17.81	54.98	3.53	5.08
Flower Stage 12, Sepals	286.2	596.91	627.41	153.86	151.1	15.03	59.41	330.31	8.88	811.66	161.53	472.2	2.21	5.28
Flower Stage 15, Carpels	21.91	219.71	127.35	11.6	53.6	2.71	7.18	21.8	12.51	310.78	29.48	43.78	2.4	3.61
Flower Stage 15, Petals	27.63	428.73	358.01	1708.95	1072.43	4.25	44.13	389.6	28.23	1421.36	506.38	519.41	2.9	3.08
Flower Stage 15, Sepals	393.31	395.73	778.45	1815.68	1022.23	3.03	61.93	471.26	21.96	1422.94	438.03	1153.38	6.41	2.81
Flower Stage 15, Stamen	30.2	328.38	243.5	1031.93	977.73	70.63	68.63	509.98	22.39	1566.7	605.43	451.98	1.51	4.59
Flowers Stage 15, Pedicels	19.58	95.85	134.65	50.18	23.3	2.15	5.8	30.85	20.45	158.81	14.33	29.61	0.96	4.96
Hypocotyl	57.21	112.06	173.71	15	53.36	1.66	10.48	17.48	22.56	4.1	15.31	73.64	3.09	6.1
Root	42.56	184.25	191.2	24.76	30.21	2.76	16.49	17.65	15.8	7.1	41.01	97.15	1.31	4.61
Rosette Leaf 2	49.29	78.69	125.3	52.71	58.41	2.33	96.86	219.58	14.19	52.13	37.3	332.06	3.01	5.63
Rosette Leaf 6	26.06	63.55	163.13	19.28	23	2.15	29.9	69.96	9.16	30.91	14.55	176.05	1.63	7.08
Rosette Leaf 10	11.6	50.48	163.81	14.91	6.93	2	14.3	21.63	8.79	23.13	10.51	92.83	0.75	6.58
Senescing Leaf	169.03	334.91	449.78	2179.18	692.76	1.71	134.91	512.08	44.86	828.81	305.33	585.35	1.45	8.55
Mature Pollen	25.96	158.96	27.45	17.59	23.58	5.45	32.56	6.01	7.28	12.48	20.71	18.46	2.01	14.95
Seeds Stage 3 w/ Siliques	22.66	137.7	103.25	151.63	85.23	2.5	29.18	54.56	16.8	185.76	131.43	76.66	1.55	4.5
Seeds Stage 5 w/ Siliques	34.19	119.08	93.6	30.6	40	5.1	366.6	623.46	92.73	101.35	26.4	82.18	2.23	2.93
Seeds Stage 7 w/o Siliques	422.05	798.68	177.31	70.08	135.7	1.65	388.23	1011.35	85.63	645.68	450.26	690.25	2.11	6.6
Seeds Stage 8 w/o Siliques	1280.1	2100.9	204.16	220.88	78.63	2.71	145.25	596.03	28.5	525.94	620.73	825.85	5.05	7.3
Seeds Stage 9 w/o Siliques	2354.54	3445.36	192.21	370.28	175.86	2.68	293.95	689.95	21.08	1108	991.3	1262.73	7.08	9.5
Seeds Stage 10 w/o Siliques	1731.18	3137.13	236.8	261.36	117.66	4.31	163	490.08	19.06	540.23	765.35	807.5	6.75	9.48
Vegetative Rosette	19.71	61.9	128.3	16.41	16.2	2.11	4.8	14.6	15.61	37.53	13.16	93.83	1.09	4.5

Figure 4.19: *ANAC032* and *ATAF1* show highest expression in mature seed stages

Developmental expression of the ATAF members (first three lanes) and other NAC TFs from the NAC-a sub-family are shown. The data is reported as absolute expression values (as reported by eFP Browser) such that higher values are shaded red as indicated by the gradient scale.

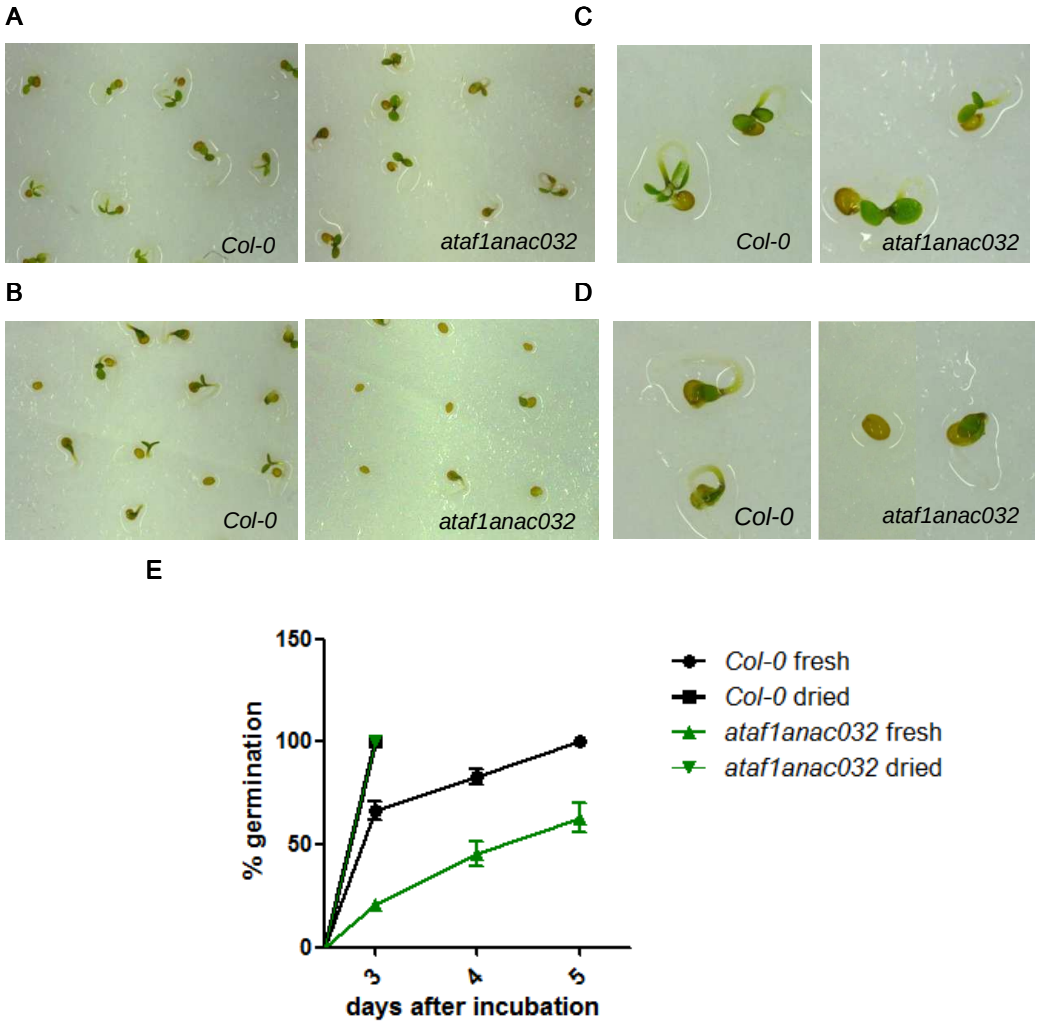


Figure 4.20: *ANAC032* and *ATAF1* are negative regulators of dormancy

About 50 completely dried (hence fully released from dormancy) (**A**) and freshly harvested seeds (**B**) were plated onto a filter paper moistened with demineralized water in Petri dishes and incubated under long day conditions. Photographs and scoring of radicle emergence was done 3 days later. **C** and **D** are representative close-up pictures of A and B respectively (**E**) Germination profiles of *Col-0* and *ataf1anac032* dried and freshly harvested seeds. Percentages are means (±SE) of two experiments each with two biological repeats.

Further, it was speculated that if seeds have high *ANAC032* expression they may not show induction of defense-related genes (since over-expression of *ANAC032* led to suppression of defense-related genes of SA, JA and JA/ET pathways, shown above). As a first indication to see if this might be true, plants containing the *JAZ10-GusPlus*

reporter construct (in the *Col-0* background) were grown until 10-15 days after siliques started appearing. The plants were then treated with MeJA and the siliques were subjected to the GUS staining procedure. As shown in Figure 4.21 fresh green siliques responded to MeJA by inducing expression of *JAZ10-GusPlus* reporter as indicated by the blue colouring. The seeds within these siliques however do not stain suggesting no induction of *JAZ10* occurs in the seeds. Yet, this is no direct evidence of the high levels of NAC TFs suppressing JA response in the seeds and more experiments need to be conducted to provide support to the hypothesis.

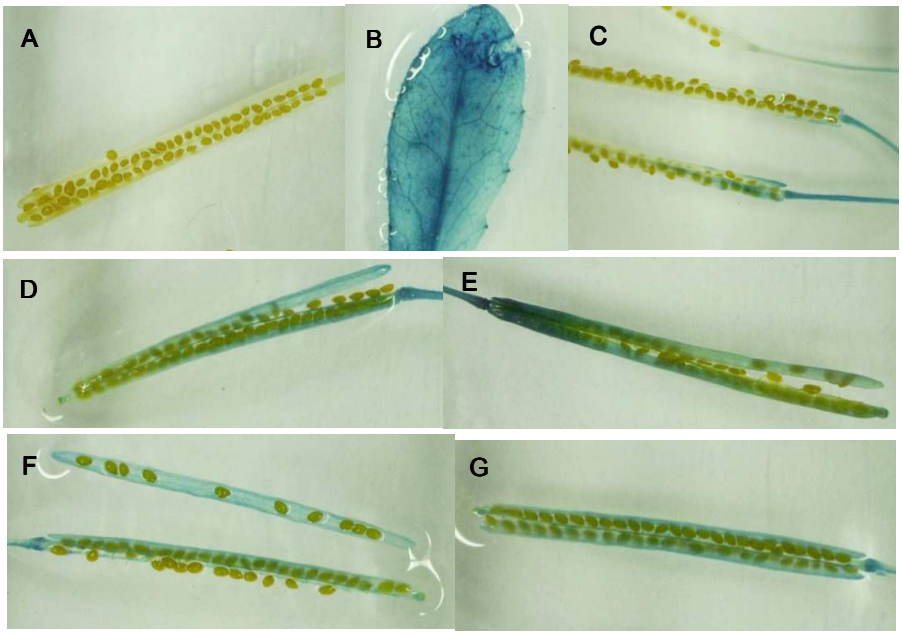


Figure 4.21: Seeds do not show JA-induced expression of *JAZ10*

JA-responsive *JAZ10-GusPlus* reporter lines were grown (long day) until 10-12 days after the first siliques were formed. The plants were then treated with 4.5μM MeJA via the gaseous phase for 6 hours, untreated plants served as controls. Siliques and leaves (control) were cut and subjected to GUS staining protocol. **A** untreated **B** JA-treated rosette leaf **C** siliques that had already started turning brown **D, E, F** and **G** green siliques cut open after staining to show unstained seeds within. *Col-0* plants were also included as controls and these did not show any staining (photographs not shown)

5. Discussion

The NAC superfamily of proteins, consisting of more than a hundred members, is a group of plant-specific transcription factors (TFs) that are involved in many different plant processes. Some NAC TFs have been identified as being necessary for proper development while others seem to play roles in response to various stresses or in plant defense (Olsen et al., 2005; Ooka et al., 2003). The ATAF subfamily, which consists of four members – *ANAC032* (At1g77450), *ATAF1* (At1g01720), *ATAF2* (At5g08790) and *ANAC102* (At5g63790) – is induced by many abiotic stress situations as well as after pathogen infection. *ATAF1* and *ATAF2* were first identified due to their ability to activate the *Cauliflower mosaic virus CaMV 35S* promoter in yeast (Xie et al., 1999). *ATAF2* is reported to be induced in response to wounding, SA, MeJA, pathogens like *Tobacco mosaic virus* and abiotic stress (Delessert et al., 2005; Wang et al., 2009a). It was recently reported that the *ATAF2* promoter can be induced upon treatment with indole-3-acetonitrile which is converted within the plant to auxin via nitrilases. ATAF2 was further found to directly regulate the expression of *NITRILASE2* (*NIT2*) gene involved in auxin biosynthesis (Huh et al., 2012). In case of *ATAF1*, however, there have been contradicting studies suggesting both positive and negative roles in biotic and abiotic stress responses (reviewed by Mauch-Mani and Flors, 2009). The remaining two members of the ATAF sub-family have not been characterized until now. Previously, we reported *ATAF1* and its closest homologue *ANAC032* as being target genes of the TGA/SCL14 complex in the detoxification pathway triggered by toxic chemicals like TIBA (Fode et al., 2008). This thesis focuses on unravelling the role played by these two NAC TFs in the detoxification pathway as well as their possible roles in defense and development.

5.1 Role of *ANAC032* and *ATAF1* in the xenobiotic-induced detoxification response

It has been shown that *ANAC032* and *ATAF1* transcript levels are four-fold more abundant in the *HA3-SCL14* overexpressing transgenic line as compared to the *scl14*

knockout mutant. Because of this and since the two NAC proteins contain putative *as-1-like* elements in their promoters (Figure 4.1 A) they are likely direct targets of the TGA/SCL14 complex. This complex recognizes *as-1-like* sequences and promotes gene expression in response to xenobiotic stress (Fode et al., 2008; Katagiri et al., 1989). Consistently, TIBA-induced and to some extent the basal expression of *ANAC032* and *ATAF1* was severely suppressed in the *tga256* triple and *scl14/33* double mutants (Figure 4.1 B). Since *ANAC032* and *ATAF1* are significantly induced after TIBA application they might be playing a role in the TGA/SCL14-triggered detoxification response (Figure 4.1). However, the NAC TFs do not seem to be essential for the complete response because unlike *tga256* and *scl14/33* which are unable to grow on TIBA-containing media, the single knockouts *anac032* and *ataf1* as well as the double knockout *ataf1anac032* germinate and grow like wild-type *Col-0* seedlings (Figure 4.2). This may be due to functional redundancy with the two other ATAF-type proteins – ATAF2 and ANAC102 – both of which contain *as-1-like* promoter elements and are induced after TIBA in a TGA/SCL14 dependent manner (Figure 4.1). Alternatively, it might be that these transcription factors regulate only a sub-branch of the TGA/SCL14-dependent detoxification program.

Contrary to *ATAF1*, functional studies on *ANAC032* have not been carried out previously and hence this present study was focused more on the characterization of *ANAC032*. Thus, microarray analysis was carried out comparing untreated wild-type plants with transgenic plants that over-expressed *35S:ANAC032*. GO term enrichment analysis indicated that of the 347 genes that were up-regulated (>2-fold, $p<0.05$) in the *35S:ANAC032* plants, 30% were associated with response to various stimuli of which chemical stimulus was the most prominent (69 genes) (Figure 4.3 A). Further, these sixty-nine genes noticeably revealed the up-regulation of many transmembrane transporters that are involved in multidrug transport. There were seven members that belonged to the MATE (**m**ultidrug **a**nd **t**oxic compound **e**xtrusion) efflux family which are known to localize to vacuoles or to the plasma membrane and play a role in the detoxification of secondary metabolites generated in plants and xenobiotics (Hvorup et al., 2003; Omote et al., 2006). The MATE

transporter *ATDTX1* (***A**rabidopsis **t**haliana **detox**ification **1***; At2g04040) which was strongly up-regulated (~12-fold) was previously reported to localize to the plasma membrane where it mediates the export of plant derived alkaloids, antibiotics and other toxic compounds such as tetraethylammonium and berberine (Li et al., 2002). The *ATMRP4* (***A**rabidopsis **t**haliana **m**ultidrug **r**esistance-associated **p**rotein **4***; At2g47800), which was moderately induced (~3-fold), belongs to the ABC group of transporters of which many are known to be involved in detoxification of cells (Kang et al., 2011). These observations suggest that *ANAC032*, in response to a chemical stimulus, induces drug transporters so as to sequester secondary metabolites or xenobiotic compounds in vacuoles or to excrete them out of cells.

Comparison of the array to a previous array that identified TIBA-induced genes in *Col-0* plants (Thesis by Dr. Julia Köster) showed around 78 genes that were commonly up-regulated according to the two arrays and 8 genes that were down-regulated in both experiments (Supplementary Tables S3 and S4). All of these genes could be potential direct or indirect targets of the NAC TFs after activation of the detoxification pathway. Among these, two genes that were strongly induced in *35S:ANAC032* plants are aldo-keto reductases – *AKR4C9* (~57-fold; At2g37770) and *AKR4C8* (~13-fold; At2g37760) – which were recently shown to be induced by various forms of stresses and proposed to play a role in detoxification of sugar-derived reactive carbonyls (Saito et al., 2013; Simpson et al., 2009). The two proteins were shown to have an inclination to reduce a wide range of substrates including 4-hydroxy-2-*trans*-nonenal, hexenal, glyoxal and methylglyoxal that are known to arise as a result of lipid peroxidation, sugar fermentation especially under stress conditions (Farmer and Davoine, 2007; Saito et al., 2013; Yadav et al., 2008). Another strongly induced oxidoreductase, *At3g04000* (~14-fold), in a study along with *AKR4C9* was implicated in the reduction of saturated and unsaturated aldehydes in chloroplasts (Yamauchi et al., 2011). In vitro studies suggested that the primary role of these oxidoreductases maybe to detoxify a range of toxic aldehydes and ketones produced during stress.

The *AKR4C9* and *At3g04000* genes along with a third gene, *At1g10585* which is a bHLH-type TF [second-most highly induced gene (~43-fold)] were selected for further analysis. All of the three genes were induced by application of TIBA in the wild-type, with induction being compromised in the single and double knockouts of the NAC TFs (Figure 4.6 A). Moreover, promoters of these three genes could be induced by ANAC032 and ATAF1, in transiently transformed protoplasts, although only when fused to the strong activating domain VP16. Additionally, all three target genes show in their promoter regions the presence of two or more putative NAC binding sites as described by Jensen et al., 2013 for ATAF1 TF (Supplementary Figure S3). This suggests that the three candidate genes are direct targets of the NAC proteins which perhaps require additional factor(s) for transcriptional activation. (Figure 4.6 B). The fact that induction of the three genes after TIBA application was suppressed in the *tga256* and *scl14/33* mutants further supports the hypothesis that these are downstream targets of the NAC TFs in the TGA/SCL14-triggered detoxification signaling cascade (Supplementary Figure S2).

Available microarray databases (Genevestigator) and previous studies suggest that *ANAC032* and *ATAF1* can be induced under a variety of stress conditions (Hruz et al., 2008; Kleinow et al., 2009). As shown in Figure 4.7, ANAC032 and ATAF1 can indeed be induced by application of various hormones and chemicals like MeJA, ABA, TIBA and ozone. They can also be induced via wounding and infection with the necrotrophic pathogen *Botrytis cinerea*. The level of induction differed depending on treatment as well as perhaps the time for which treatment was applied. The three target genes *AKR4C9*, *bHLH585* and *At3g04000* were also induced by these different treatments with a pattern quite consistent with that observed for the NAC TFs (Figure 4.7 D-F). Similar to TIBA application, the induction of *ANAC032* seemed to require the TGA/SCL14 complex after wounding (Figure 4.8 A). Consistently, the three target genes which were all strongly induced after wounding showed compromised induction in the *ataf1anac032* and the *tga256* and *scl14/33* mutant plants (Figure 4.8 B and C).

The compromised induction of the target genes seen in the *ataf1anac032* mutant plants be it after TIBA treatment or wounding, was never completely abolished. This was especially observed in case of *bHLH585* which showed similar levels of induction after wounding in the double knockout and wild-type plants which would suggest that its expression is not fully dependent on the NAC TFs. Nonetheless, it is possible that the two other closely related ATAF-type NAC factors (ATAF2 and ANAC102) may function redundantly and knockout of all four may be required to perceive differences with the wild-type (since ATAF2 and ANAC102 are also induced under these situations; Figure 4.1 B, Figure 4.7 A, and Figure 4.8 D).

All of the above observations suggest that *ANAC032* and *ATAF1* may directly target many genes and activate a downstream response involving transcription factors like *bHLH585* and enzymes (like *AKR4C9* and *At3g04000*) and maybe other proteins like drug transporters etc. However, this response is not specifically activated after stimulus from a xenobiotic compound like TIBA. Instead we propose that the NAC TFs are more likely to be activators of a general stress response triggered under different stress situations and part of their function is to induce genes that will then help in detoxification of toxic compounds generated under stress conditions.

5.2 Role of *ANAC032* in phytohormone-mediated defense responses

Previously in our lab, it was observed that over-expression of *ANAC032* led to the suppression of MeJA-induced *PDF1.2* and *VSP2* expression (Dr. Julia Köster, PhD thesis). This suppression of *VSP2* by *35S:ANAC032* was confirmed in this study and it was further observed that over-expression of *35S:ATAF1* had the same effect whereas *ataf1anac032* knockout mutant induced the genes to similar levels like wild-type (Figure 4.12 A). Moreover, the ACC-induced expression of *PDF1.2* was compromised by over-expressing the two NAC TFs with no obvious effects observed in *ataf1anac032* plants (Figure 4.12 C). Further, these suppressive effects could be seen at the level of *MYC2* and *ORA59* genes which act up-stream of *VSP2* and *PDF1.2* respectively (Figure 4.12 B and D). The antagonism between SA-JA or SA-

ET signaling pathways is a well-established phenomenon with reports of several different proteins playing a role in the cross-talk, including the TGA factors, leading to suppression of downstream defense genes (see Introduction section 1.2.3; Van der Does et al., 2013; Pieterse et al., 2009; Zander et al., 2010, 2012). To check if this antagonism could explain the suppression observed in the *35S:ANAC032* plants, cross-talk experiments were carried out with the *ataf1anac032* mutant plants. However, the suppressive effects of the cross-talk observed in *ataf1anac032* were as prominent as in wild-type (Figure 4.13 A). This perhaps suggests that the NAC proteins do not play a role in the SA-ET cross-talk; alternatively, their role may be minor and other proteins carrying out the cross-talk leave no discernible effects to be detected in the knockout plants. For instance, glutaredoxins like GRX480 was found to interact with TGA factors and suppress the expression of ORA59 (Ndamukong et al., 2007; Zander et al., 2012). Thus two redundant mechanisms, one involving the GRX proteins and another involving the NAC TFs, may exist for the SA-ET antagonism due to which knockout of either one shows no phenotype.

Surprisingly, the NAC over-expressing plants also showed a compromised induction of the SA-inducible *PR1* gene after SA treatment (Figure 4.13 B). There have been previous contradictory reports about effects of *ATAF1*-overexpression on the induction of defense genes. One report described a down-regulation of both *PR1* and *PDF1.2* (Wang et al., 2009b) while another claimed that *PR1* expression was induced by ATAF1 with no effects on *PDF1.2* (Wu et al., 2009). Another study has also shown down-regulation of *PR* genes by *ATAF2*, one of the four ATAF proteins (Delessert et al., 2005). In this current study, over-expression of *ANAC032* and *ATAF1* seemed to suppress JA-, ACC-, as well as SA-responsive genes.

To see if suppression is due to altered signaling or defective accumulation, hormone levels were measured in plants treated with ozone. Measurement of hormone levels in *35S:ANAC032* and *35S:ATAF1* were not stringently reproducible between three independent experiments (Figure 4.15 and 4.16) leading to inconclusive data. However, the basal total SA content was consistently higher in both the NAC-over expressing plants as compared to wild-type (Figure 4.15 A and B) suggesting that

these plants are capable of synthesizing SA. The glucosylated form of SA, SA-2-O-β-D-glucoside (SAG) is also greatly elevated in *35S:ANAC032* plants (Figure 4.16 C and D) which may be due to the high expression of *UGT74F2* gene (as indicated by the microarray, ~19-fold) that is responsible for the conversion of SA to SAG (Lim et al., 2002). However the reasons for high SAG needs to be confirmed especially because a previous report indicates that contrary to expectation, over-expression of *UGT74F2* leads to reduced levels of both free SA and SAG and instead shows enhanced levels of MeSA and MeSAG leading to compromised immune response (Song et al., 2008). Nonetheless, the high *UGT74F2* expression could in principle lead to increased accumulation of inactive forms of SA and thus explain the reduced *PR1* expression observed after SA treatment.

The measured levels of JA and its related metabolites were even more difficult to comprehend. In one experiment *35S:ANAC032* showed increased amounts of JA in basal and induced conditions while in the second experiment only induced levels were significantly higher than wild-type (Figure 4.17 A and B). In the case of amino acid conjugate JA-Ile, one experiment showed higher basal levels only in *35S:ANAC032* while the second experiment showed high basal levels only in *35S:ATAF1*. On the other hand, in both experiments, the induced levels of JA-Ile were significantly higher in both over-expressing plants compared to wild-type (Figure 4.17 E and F). The hydroxylation product 11_12-OH-JA showed similar pattern to measured JA levels with *35S:ANAC032* containing significantly abundant basal and induced levels of the metabolite (Figure 4.17 G and H). A third ozone-experiment conducted showed differences again leading to no concrete conclusions. In all experiments however the knockout *ataf1anac032* consistently behaved like wild-type unlike the over-expressers that showed different results between experiments. This is almost similar to the above mentioned controversial reports about the suppression of gene expression and perhaps these discrepancies are due to environmental factors influencing the regulation by NAC proteins. Nevertheless, the ability to synthesize or accumulate the hormones (SA, JA and related metabolites) after induction was not compromised in *35S:ANAC032* or *35S:ATAF1* plants, suggesting that the suppressive effects are probably targeted at signaling

rather than inactivation of the hormones. Instead, in some experiments over-expression of the NAC TFs seemed to positively regulate the biosynthesis of SA and JA.

Over the years, JA has been established as the wound hormone although it is now clear that other signaling pathways contribute to the wound response (see Introduction section 1.3). The *ANAC032* induction observed after wounding does not seem to depend on JA since both JA-biosynthesis mutant *dde2-2* as well as JA-signaling mutant *coi1-t* accumulates *ANAC032* to similar levels as wild-type (Figure 4.10). Moreover this induction seems to be transient (peaking within 90 minutes) and restricted to the locally wounded tissue with minor or no induction detected in unwounded systemic leaves (Figure 4.11 A and B). Although JA rapidly accumulates in local as well as systemic leaves after wounding, its role in local leaves is poorly understood with most effects being evident in the systemic tissue where it establishes defense response against further attack by herbivores or pathogens. In contrast, locally-induced genes are speculated to be involved in wound healing and repair in addition to protection against water loss and invasion by pathogens (Delessert et al., 2004; Glauser et al., 2008). Since wound-induced *VSP2* is suppressed in the *35S:ANAC032* and *35S:ATAF1* plants with no difference between *ataf1anac032* and wild-type, the two ATAF members do not seem to contribute to establish JA-induced systemic defense response (Figure 4.9). Further, because ANAC032 strongly induces similar genes after activation of detoxification pathway and wounding (e.g. *AKR4C9*, *bHLH585*, Figure 4.6 and Figure 4.8), it is reasonable to speculate that here too at least part of its function is as an activator of a more general stress response helping perhaps in the local wound healing process.

The ABA pathway which is central to the abiotic stress response is also known to have antagonistic effects on the defense pathways (see Introduction 1.2.3; Cao et al., 2011; Mauch-Mani and Mauch, 2005; Moeder et al., 2010). *ATAF1* was previously reported to negatively regulate ABA-inducible genes (that normally play a role in abiotic stress response) and act as an attenuator of ABA signaling upon infection by *Blumeria graminis f.sp. hordei* (Jensen et al., 2008). However basal levels of ABA

were found to be low in *ataf1* mutants suggesting a positive role for ATAF1 in ABA biosynthesis. This pointed to an antagonistic function of ATAF1 and it was postulated to act as a switch between abiotic stress tolerance and defense responses (Mauch-Mani and Flors, 2009). To see if the suppression of *PDF1.2* in plants ectopically expressing the NAC TFs can be explained by antagonistic interaction with ABA, cross-talk experiments were carried out by treating plants with ABA and ET. However, like wild-type the *ataf1anac032* plants also displayed suppression of *PDF1.2* due to antagonistic action of ABA indicating that the NAC TFs do not contribute to the ABA-ET cross-talk (Figure 4.13 C). However, as postulated in case of SA-ET cross-talk, the ABA-ET antagonism may also be mediated in more ways than one, making it difficult to observe a phenotype in the *ataf1anac032* plants. Moreover, ectopic expression of *ANAC032* also led to suppressed induction of ABA-inducible *COR78* gene (Figure 4.16 D). Further, it was observed that *35S:ATAF1* plants contain significantly higher levels of ABA (Figure 4.17) and its inactive form ABA-GE (data not shown) under basal as well as induced conditions after ozone treatment. Consistent to this, the single knockout *ataf1* showed compromised accumulation of ABA after ozone treatment although the double knockout *ataf1anac032* displayed wild-type-like levels (Figure 4.17). This finding that over-accumulation of *ATAF1* leads to increased accumulation of ABA is consistent with a recent report that ATAF1 positively regulates ABA biosynthesis through the transcriptional activation of the biosynthesis gene *NCED3* (Jensen et al., 2013). *ANAC032*, however, does not seem to function redundantly in this case although after ozone treatment it may be contributing slightly to increased ABA accumulation (Figure 4.17).

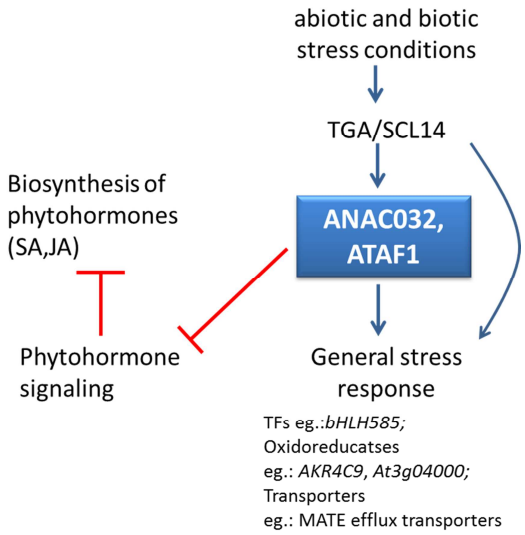


Figure 5.1: Schematic representation of role of ANAC032 and ATAF1 in stress response

ANAC032 and *ATAF1* are induced within the plant under diverse stress conditions and in turn it induces a number of genes which may be part of a general stress response. The induction of the NAC TFs seems to depend upon the activation of the TGA/SCL14 complex depending on the stress cue. The NAC TFs may also suppress phytohormone-induced defense responses and directly/indirectly promote hormone biosynthesis via yet unknown mechanism.

Based on the current and reported studies a model may be proposed for *ANAC032* and *ATAF1* in response to abiotic and biotic stress (Figure 5.1). The ATAF TFs are induced under diverse abiotic as well as biotic stress conditions and in some cases the induction may be rapid (within 3 hours) and transient. Moreover the induction of *ANAC032* requires the TGA/SCL14 complex not only under chemical stress but also under stress situations like wounding. Induced *ANAC032* most likely triggers a general stress response rather than stress-specific response, directly inducing several genes like oxidoreductases and other TFs. It also may induce directly or indirectly a number of transmembrane transporters including multidrug efflux transporters which play a role in the sequestration/excretion of toxic compounds.

The NAC TFs also seem to modulate phytohormone-mediated defense responses (Figure 5.1). The study indicates that all defense (SA, JA and ET) and abiotic stress

(ABA) signaling is suppressed to some degree by the over-expression of both NAC TFs. On the other hand, the biosynthesis of hormones seems to be positively regulated by the NAC TFs. However, this could be an indirect effect stemming from a suppressed signaling cascade leading to a lack of feed-back control on biosynthesis by components of the signal transduction. The mechanism by which suppression of hormone-induced gene expression is brought about remains unclear. One possibility is that the NAC TFs induce a regulator which is then able to suppress all hormone-induced defense mechanisms within the plant. However, microarray analysis of differentially regulated genes in *35S:ANAC032* plants did not provide any clue for the identity of such a repressor. On the other hand, such a repressor may accumulate at the protein level leading to suppression. This has been observed in the case of gibberrellic acid (GA) pathway, where DELLA repressors accumulate thereby suppressing GA signaling and thus plant growth (Achard and Genschik, 2009). As mentioned above, only the fused NAC TFs were able to activate the promoters of the target genes, which suggests requirement of co-activators in the system. Thus a second likelihood is that, the NAC TFs may act similarly by recruiting co-repressors to promoters of key regulators of the phytohormone pathways thus suppressing them. A further possibility is that the NAC TFs themselves directly interact and functionally block players of phytohormone-mediated responses. Moreover elements such as environmental factors may influence the positive or negative regulatory functions of the NAC TFs which may be the cause of inconsistencies observed in this and reported studies. Alternatively, the suppressive effects on defense pathways could be artifacts of constitutive over-expression of the NAC TFs. The expression levels found in the transgenic plants may never be achieved for long durations under natural circumstances since studies suggest that ATAF members are rapidly and transiently induced by some hormones and treatments (Wu et al., 2009, wounding experiments in this study).

5.3 Role of *ANAC032* in plant developmental processes

Transgenic *Arabidopsis* plants carrying overexpression constructs for either *ANAC032* or *ATAF1* showed severe developmental defects (Figure 4.18). This included dwarfism crinkled and upward curled leaves showing early yellowing, sterility and delayed or absent flower initiation. The severity of these irregularities seemed to depend on the expression levels of the transgene with stronger expression leading to more severe effects. Similar phenotypic defects have been characterized earlier for transgenic plants over-expressing *ATAF1* (Jensen et al., 2013; Kleinow et al., 2009; Wu et al., 2009) or *ATAF2* (Huh et al., 2012). Microarray analysis and qRT-PCR revealed that *35S:ANAC032* plants showed an increased expression of *FLOWERING LOCUS C* (*FLC*) (Figure 4.5, Table 4.1), a MADS box transcriptional regulator which may explain the delayed flowering phenotype (Figure 4.18 B). High *FLC* activity in plants leads to late flowering because FLC directly suppresses expression of floral inducers *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*) (Hepworth et al., 2002; Searle et al., 2006). Recently, a study showed that *FLC* delays the juvenile-to-adult vegetative transition and affects traits associated with vegetative phase change. It was shown that plants over-expressing *FLC* contain a higher number of juvenile leaves which are characterized by rounder leaves and by decreased number of abaxial trichomes compared to adult leaves (Willmann and Poethig, 2011). Consistent to this, the *35S:ANAC032* plants also show more juvenile leaves that are rounder and have less abaxial trichomes (Figure 4.18 C and D). In summary, the delayed juvenile to adult transition and the delayed flowering of *35S:ANAC032* plants might be due to the elevated *FLC* expression. However, the slow growth must be due to other mechanisms since *35S:FLC* plants grow normally (Michaels and Amasino, 2001). Even though *FLC* does not seem to be a direct target of ANAC032 or ATAF1 (Figure 4.6 B), it seems to be indirectly induced by these NAC proteins, a process that might be of physiological relevance in seeds, where high levels of ANAC032 and ATAF1 are expressed (see below).

Since ANAC032 and ATAF1 are highly expressed in mature seeds (Figure 4.19), we tested whether they affect the establishment of seed dormancy. Seed dormancy is the mechanism to prevent germination under unsuitable ecological conditions when the probability of survival is low. The transition between dormancy and germination is very critical and is regulated by hormones whose degradation and synthesis respond to external environmental factors. Abscisic acid and gibberellic acid (GA) play a dynamic role in maintaining the equilibrium between dormancy and germination (del Carmen Rodriguez-Gacio et al., 2009). ABA usually promotes dormancy induction and maintenance while GA promotes germination. The two hormones act antagonistically to each other. The master regulators of this process are the DELLA proteins RGL1, RGL2 and RGL3, which stimulate ABA synthesis. ABA acts in a feed-forward mechanism to maintain RGL transcription. The ABA-induced signal cascade negatively regulates GA synthesis. Signals that break dormancy lead to the degradation of ABA through ABA-deactivating enzymes like CYP707A2 and to reduced transcription of *RGL1,2,3*. This leads to the release of suppression on GA biosynthesis and increased GA content, which in turn favours degradation of RGL1,2,3, so that ABA biosynthesis is strongly impaired (Ariizumi et al., 2011; Footitt et al., 2011; Lee et al., 2010; Seo et al., 2006) (see further Figure 5.2). Since ABA can significantly induce *ANAC032*, we speculated whether ABA-induced *ANAC032* plays a role in regulation of seed dormancy.

In *Arabidopsis*, freshly harvested seeds are dormant and environmental cues like light, low temperature and after-ripening break dormancy (Koornneef et al., 2002). In order to monitor different levels of dormancy, freshly harvested seeds were exposed to two days in the cold to partially relieve the dormant state. Indeed, freshly harvested and stratified seeds of *ataf1anac032* showed less germination (~20%) as compared to wild-type (80%) three days after placing them on wet filter paper (Figure 4.20). This shows that *ANAC032* and *ATAF1* are negative regulators of seed dormancy. In contrast, germination rates were similar when seeds were exposed to after ripening and stratification, indicating that germination per se is not affected. A previous study had stated that *ataf1* knockout plants showed a very low germination rate while over-expression of *ATAF1* led to increased germination rate compared to

wild-type (Wu et al., 2009). However, the paper does not describe whether dormant or non-dormant seeds were used for the analysis. Our microarray analysis has revealed candidate genes which might confer dormancy when being expressed in seeds. Known negative-regulator of ABA-induced seed dormancy, *CBL-INTERACTING PROTEIN KINASE 3* (*CIPK3*) (Kim et al., 2003; Pandey et al., 2008) is induced (~2-fold) in *35S:ANAC032* plants. Other candidates include *INOSITOL(1,4,5)P3 5-PHOSPHATASE II* (*AT5PTASE2*; ~4-fold induction) which when overexpressed is reported to be insensitive to ABA in germination assays (Gunesekera et al., 2007) and *ARABIDOPSIS THALIANA ENT-KAURENE OXIDASE 1* (*ATKO1*; ~3.5-fold induction) which is a GA-biosynthesis gene up-regulated during seed germination (Ogawa et al., 2003). Further, it was recently shown that protein levels of ABI3 and ABI5, which are positive regulators of ABA-induced seed dormancy, are higher in the *scl14* mutant (Bassel et al., 2011). Hence it is conceivable that *ANAC032* expression in mature seeds is dependent on the TGA/SCL14 complex which would explain the lower germination rate of freshly harvested *scl14* mutants and the *ataf1anac032* double mutant. Moreover, *FLC* has also been reported to play a role in the developmental transition towards germination. The FLC regulation of germination involves *FT* and *SOC1* which directly or indirectly promotes the ABA catabolic pathway (via *CYP707A2*) and the gibberellin biosynthetic pathway (via *GA20ox1*) in seeds (Chiang et al., 2009). Even though *FLC* does not seem to be a direct target of ANAC032 (Figure 4.6 B), it may be indirectly induced by the NAC protein to high degree during seed maturation. Lastly, since *ANAC032* expression is very high in seeds we speculated that, if ANAC032 can indeed suppress phytohormone signaling then seeds should not show up-regulation of defense-related genes, for example MeJA-induced *JAZ10* expression. Indeed, as seen in Figure 4.21, when *JAZ10-GusPlus* reporter lines were treated with MeJA, green siliques stained blue indicating activation of the *JAZ10* promoter while the seeds within them remained unstained. However, it remains to be analyzed whether this observation is undeniably due to high expression of *ANAC032*.

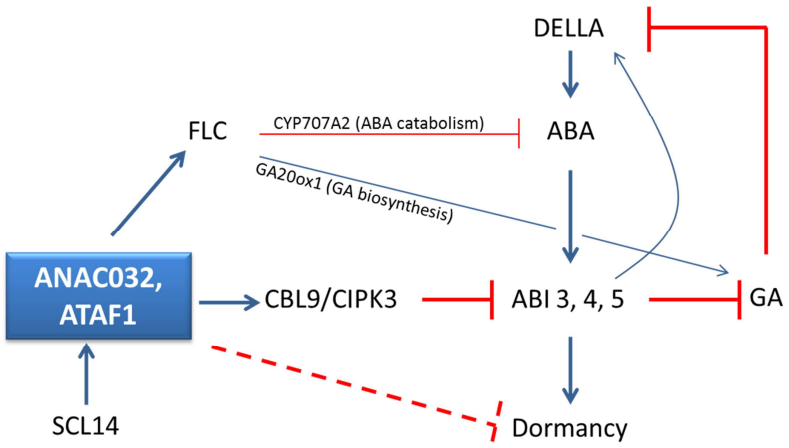


Figure 5.2: Schematic representation role of ANAC032 and ATAF1 in seed dormancy

ANAC032 and ATAF1 show high expression within mature seeds where it negatively influences seed dormancy (observed phenotype is indicated by dotted line). This may be via induction of CIPK3 which is known to negatively influence dormancy, or possibly via *FLC* also known to influence the ABA-GA balance in favour of germination. The TGA/SCL14 is likely to play a role in the induction of *ANAC032* within seeds. The dotted line represents the actual observation.

Thus, apart from its role in stress response, a second important function of the NAC TFs can be imagined in the mature seed (Figure 5.2) where they are highly expressed. Here, *ANAC032* seems to negatively regulate seed dormancy and promote germination. This may be achieved via induction of *CIPK3* which has been shown to negatively regulate dormancy. Further, studies indicate SCL14 promotes germination, probably by an indirect influence on protein accumulation of positive regulators of dormancy such as ABI3 and ABI5. Therefore, we propose that SCL14-induced *ANAC032* may further induce genes like *CIPK3* which through its kinase activity may deactivate or alter the stability of ABI3 and ABI5. Promotion of seed germination by *ANAC032* may be partly achieved via *FLC* which promotes transition to seed germination by promoting both ABA catabolism and GA biosynthesis. Additionally, induction of GA biosynthesis genes in the *35S:ANAC032* plant indicates that there may be more direct ways in which *ANAC032* influences seed dormancy.

As mentioned earlier, the ATAF sub-family members are proposed to function redundantly as it is co-expressed under varied conditions (Kleinow et al., 2009). Indeed in the current study knockout of *ATAF1* and *ANAC032* alone did not lead to a complete suppression of expression of target genes. Moreover, *ataf1anac032* behaved like the wild-type in most cases when looking at phytohormone-mediated gene expression. This could be due to the functional redundancy between the ATAF TFs and a future knockout of all four members may be required to observe a stronger and clearer phenotype. However in some respects all four proteins do not seem to be redundant. For example, over-expression of *ATAF1* positively regulates ABA synthesis while *ANAC032* overexpression shows only minor and/or insignificant increase in ABA accumulation (Figure 4.16). On the other hand, JA synthesis seems to be positively regulated only by *ANAC032* and not by *ATAF1* which behaves like the wild-type (Figure 4. 15 A and B). The function of *ANAC032* and *ATAF1* may be redundant in the mature seed as indicated by *ataf1anac032* mutant, however this function may not be true of *ANAC102* since it does not show high expression in seeds and instead shows highest expression in senescent leaves (from eFP browser). Thus more careful and stringent studies need to be carried out in future to dissect out the various redundant and non-redundant functions of the ATAF-type transcription factors.

6. Summary

In higher plants, xenobiotic chemicals induce transcriptional activation of genes involved in their detoxification. A generally accepted concept explaining gene regulatory networks is that, activation of pre-existing primary transcription factors regulates transcription of secondary transcription factors that in turn induce genes that execute the appropriate response. In the detoxification program, TGA transcription factors and their transcriptional co-activator SCL14 bind to promoters containing *activation sequence-1* (*as-1*)–like cis-elements. Microarray analysis of plants containing either lower or higher amounts of SCL14 had identified two NAC transcription factors – *ANAC032* and *ATAF1* – whose expression was affected by SCL14 (Fode et al., 2008). Thus, these are candidate secondary transcription factors that lead to the expression of downstream genes. In the current study, a microarray of transgenic *35S:ANAC032* plants was compared to a previous array that had identified genes that were activated by the xenobiotic TIBA. Seventy-eight genes were up-regulated upon TIBA treatment or ectopic expression of *ANAC032* and were thus potential direct or indirect target genes of the NAC TFs in the detoxification response. Three such potential target genes – *AKR4C9*, *bHLH585* and *At3g04000* – were found to be induced by TIBA in a TGA/SCL14-dependent manner with induction being compromised in varying degrees in the *ataf1* and *anac032* single knockout and *ataf1anac032* double knockout plants. Additionally, transient expression assays indicated that the promoters of the three genes could be induced by ANAC032 and ATAF1, although only when fused to the strong activating domain VP16. High SCL14/TGA-dependent induction of NAC TFs and the three target genes, *AKR4C9*, *bHLH585* and *At3g04000* was also observed upon wounding. This wound response was not dependent on the plant hormone jasmonic acid.

Ectopic expression of the two NAC TFs was also found to suppress SA-, JA/ET-, JA- and ABA- responsive genes. Since their expression is also triggered by these hormones, they are candidates to mediate the antagonism between the corresponding pathways. This could not be confirmed using the *ataf1anac032* double knock out, which might be due to the redundancy with the two related transcription factors ANAC102 and ATAF2 or NAC-independent mechanisms.

Lastly, ectopically expressed ANAC032 and ATAF1 led to developmental defects including dwarfism, curled leaves showing early yellowing and delayed or absent flower initiation. The latter may be due to increased expression of *FLC* observed in the *35S:ANAC032* and *35S:ATAF1* plants. Consistently, these plants show increased juvenility which is characteristic of plants over-expressing the *FLC* gene. As revealed by increased dormancy of *ataf1anac032* seeds, ANAC032 and ATAF1 seem to negatively regulate seed dormancy. The molecular basis of this regulation needs to be carefully studied in the future. However, clues may be provided by the microarray which indicated that *35S:ANAC032* plants show an up-regulation of genes related to negative regulation of seed dormancy. To sum up, the ATAF-type NAC TFs seem to have two major roles, one in modulating responses under stress conditions and the other in regulating seed dormancy.

7. References

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Acknowledgement

Completion of this doctoral thesis was possible only due to the help and support from several people to whom I shall forever be very grateful.

First and foremost I would like to offer my sincere gratitude to my supervisor Prof. Dr. Christiane Gatz for giving me the opportunity to work in her lab and on this topic. She has been a wonderful guide, encouraging at all times while patiently and critically guiding me in taking this project forward.

I wish to express my heartfelt thanks to my thesis committee and examiners Prof. Dr. Volker Lipka, Prof. Dr. Ivo Feussner, PD Dr. Thomas Teichmann, Jr. Prof. Cynthia Gleason and Prof. Dr. Andrea Polle for their constructive discussions towards the progress of this work. I sincerely appreciate the insightful suggestions and discussions of Dr. Corinna Thurow throughout this project.

I would also like to thank Prof. Ivo Feussner and Dr. Tim Iven for doing the metabolite measurements.

I would like to convey my warm and most sincere gratitude to the technical support provided by Ronny, Anna and Larissa. They all have been immensely helpful and I have always felt that they do such a wonderful job that working in the laboratory becomes considerably easy! I would like to say special thanks to Guido who is always ready to help with whatever problems or requests that you take to him. And of course, I owe a very big thanks to all my present and past colleagues - Mark, Martin, Anjali, Alex, Johanna, Li-Jun, Ning, Armin, Joachim, Sonja, Fred and Natthanon. It has been an honour and pleasure to know and work with you all. I could not have asked for a better (or more fun) working environment!

I would also like to acknowledge all my lovely friends, here and from back home, for being the wonderful, understanding and caring friends that they are.

My parents have been my inspiration throughout life and I am greatly indebted to them and my whole family who have unconditionally loved, supported and believed in me. I thank my dear husband, Nishit, for encouraging me to pursue my interests even though it meant that we would be miles apart!

9. Supplementary Material

Table S1: List of genes up-regulated in *35S:ANAC032* plants (>2-fold; P<0.05)

No.	Gene code	Symbol	Description	*35S:ANAC032/ Col-0* (fold induction)	p-value
Cell wall related					
1	AT1G26770	ATEXPA10, ATHEXP, ALPHA 1.1	ARABIDOPSIS THALIANA EXPANSIN A 10; structural constituent of cell wall	7.724935	0.000106
2	AT4G30290	XTH19	XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 19; hydrolase	4.89963	0.001922
3	AT5G34940	AtGUS3	Arabidopsis thaliana glucuronidase 3; beta-glucuronidase	4.183797	0.00031
4	AT5G06860	PGIP1	POLYGALACTURONASE INHIBITING PROTEIN 1; protein binding	3.653244	0.001729
5	AT3G43270		pectinesterase family protein	3.565157	3.57E-06
6	AT4G14130	XTR7	XYLOGLUCAN ENDOTRANSGLYCOSYLASE 7; hydrolase	3.401027	0.001132
7	AT1G28600		lipase, putative	3.353894	0.003991
8	AT1G32960	SBT3.3	identical protein binding / serine-type endopeptidase	3.331092	0.014816
9	AT4G19410		pectinacetylesterase, putative	2.724269	0.000827
10	AT5G06870	PGIP2	POLYGALACTURONASE INHIBITING PROTEIN 2; protein binding	2.528479	0.003115
11	AT3G45970	ATEXLA1, ATHEXP BETA 2.1	ARABIDOPSIS THALIANA EXPANSIN-LIKE A1	2.312124	3.38E-05
12	AT1G32170	XTR4, XTH30	XYLOGLUCAN ENDOTRANSGLYCOSYLASE 4; hydrolase	2.250298	0.003565
13	AT3G55430		glycosyl hydrolase family 17 protein	2.219432	0.002884
14	AT2G01850	EXGT-A3, XTH27	hydrolase, acting on glycosyl bonds	2.186094	0.00019
15	AT3G14060		Unknown function	2.008632	0.000479
16	AT4G11320; AT4G11310		cysteine proteinase, putative	3.660721	0.026587
Glucosyltransferase					
17	AT2G43820	UGT74F2, SAGT1	UDP-glucose:4-aminobenzoate acylglucosyltransferase	18.93009	7.38E-05
18	AT4G15490	UGT84A3	UDP-glycosyltransferase/ sinapate 1-glucosyltransferase	12.35113	8.21E-06
19	AT1G05680	UGT74E2	UDP-glucosyl transferase family protein	9.512387	7.81E-05
20	AT3G11340	UGT76B1	UDP-glucosyl transferase family protein	8.511282	0.005196
21	AT3G46660	UGT76E12	quercetin 3-O-glucosyltransferase/ quercetin 7-O-	7.649356	0.002896

			glucosyltransferase		
22	AT3G46670	UGT76E11	quercetin 3-O-glucosyltransferase	4.685537	0.002451
23	AT2G30140	UGT87A2	UDP-glucosyl transferase family protein	3.745069	0.000208
24	AT2G36750	UGT73C1	cis-zeatin O-beta-D-glucosyltransferase/ trans-zeatin O-beta-D-glucosyltransferase	3.558715	0.015248
25	AT2G36970		UDP-glucosyl transferase family protein	2.825846	0.000183
26	AT1G70090	GATL9, LGT8	GLUCOSYL TRANSFERASE FAMILY 8; polygalacturonate 4-alpha-galacturonosyltransferase	2.343912	0.002902
27	AT1G05560	UGT75B1	UDP-glycosyltransferase/ abscisic acid glucosyltransferase	2.219308	0.038063
28	AT2G36780; AT2G36770		UDP-glucosyl transferase family protein	4.159371	0.001763
Kinase activity					
29	AT5G51830		pfkB-type carbohydrate kinase family protein	3.66412	0.00038
30	AT5G11020		ATP binding / protein kinase	3.443754	0.000584
31	AT5G26570	PWD, OK1, ATGWD3	carbohydrate kinase/ phosphoglucan, water dikinase	2.926173	0.009493
32	AT3G04810	ATNEK2	NIMA-RELATED KINASE 2; ATP binding / protein kinase	2.924224	7.36E-05
33	AT2G26980	CIPK3	CBL-INTERACTING PROTEIN KINASE 3;	2.343199	0.001401
34	AT2G16790		shikimate kinase family protein	2.239525	0.008115
35	AT4G37250		leucine-rich repeat family protein / protein kinase	2.085429	0.000253
36	AT3G25560	NIK2	NSP-INTERACTING KINASE 2	2.034801	0.000999
37	AT4G36450	MPK14	Mitogen-activated protein kinase 14; MAP kinase	2.031579	0.000177
Lipid metabolism					
38	AT1G76470		3-beta-hydroxy-delta5-steroid dehydrogenase/ cinnamoyl-CoA reductase	6.669548	0.001012
39	AT5G14180	MPL1	MYZUS PERSICAE-INDUCED LIPASE 1	5.611732	0.014726
40	AT1G54000; AT1G54010		myrosinase-associated protein,	3.016294	0.003784
41	AT5G01870		lipid transfer protein, putative	2.630331	0.001856
42	AT4G16760	ACX1	ACYL-COA OXIDASE 1	2.619378	0.000233
43	AT5G07010	ST2A	SULFOTRANSFERASE 2A; hydroxyjasmonate sulfotransferase	2.545133	0.038603
44	AT4G30140	CDEF1	CUTICLE DESTRUCTING FACTOR 1, GDSL-motif lipase/hydrolase family protein	2.534671	0.006042
45	AT5G45950		GDSL-motif lipase/hydrolase family protein	2.48638	0.008911
46	AT5G45670		GDSL-motif lipase/hydrolase family protein	2.369604	0.034023

47	AT1G76090	SMT3	STEROL METHYLTRANSFERASE 3; S-adenosylmethionine-dependent methyltransferase	2.140516	0.000637
48	AT2G38180		GDSL-motif lipase/hydrolase family protein	2.102785	0.009514
49	AT4G34250	KCS16	3-KETOACYL-COA SYNTHASE 16; acyltransferase	2.010724	0.043582
Organic acid catabolism					
50	AT3G45300	IVD	ISOVALERYL-COA-DEHYDROGENASE	5.084359	0.000157
51	AT4G33150		lysine-ketoglutarate reductase/saccharopine dehydrogenase bifunctional enzyme	4.641545	0.000616
52	AT3G04520	THA2	Threonine Aldolase 2	2.717481	0.000128
53	AT3G22200	POP2, GABA-T, HER1	POLLEN-PISTIL INCOMPATIBILITY 2; 4-aminobutyrate transaminase	2.643913	0.00629
54	AT2G02000; AT2G02010	at2g02000: GAD3 at2g02010: GAD4	glutamate decarboxylase; calmodulin	2.370822	0.003104
55	AT3G58750	CSY2	citrate synthase 2	2.034736	0.030029
Oxidoreductase activity					
56	AT2G37770	AKR4C9	aldo/keto reductase family protein	56.89305	7.81E-07
57	AT1G62570	FMO GS-OX4	FLAVIN-MONOOXYGENASE GLUCOSINOLATE S-OXYGENASE 4	19.91268	1.43E-06
58	AT3G04000		short-chain dehydrogenase/ reductase (SDR) family protein	13.73018	0.000384
59	AT2G38380; AT2G38390	at2g38380: (PER22)	at2g38380: peroxidase at2g38390: peroxidase, putative	13.32886	0.001039
60	AT2G37760	AKR4C8	aldo/keto reductase family protein	12.63507	2.64E-05
61	AT2G29370		tropinone reductase, putative	10.33212	6.23E-06
62	AT1G77120	ADH1	ALCOHOL DEHYDROGENASE 1	7.962909	0.000243
63	AT1G17020	SRG1	SENESCENCE-RELATED GENE 1; oxidoreductase, acting on diphenols and related substances as donors	7.01945	0.00285
64	AT5G24910	CYP714A1	electron carrier/ heme binding / monooxygenase/ oxygen binding	6.44529	1.64E-05
65	AT1G17745	PGDH	3-PHOSPHOGLYCERATE DEHYDROGENASE	6.444687	9.94E-05
66	AT1G63340; AT1G62580		flavin-containing monooxygenase family protein	6.124668	0.000401
67	AT2G34810		FAD-binding domain-containing protein	5.385956	0.000127
68	AT1G54100	ALDH7B4	Aldehyde Dehydrogenase 7B4	4.519785	0.000288

69	AT3G03470	CYP89A9	electron carrier/ heme binding / monooxygenase/ oxygen binding	4.403794	0.004824
70	AT1G09420	G6PD4	GLUCOSE-6-PHOSPHATE DEHYDROGENASE 4	4.039865	0.001239
71	AT1G60730		aldo/keto reductase family protein	3.834442	0.00119
72	AT5G19440		cinnamyl-alcohol dehydrogenase, putative	3.728064	0.002512
73	AT1G21400; AT5G34780		at5g34780: dehydrogenase E1 component family protein at1g21400: 2-oxoisovalerate dehydrogenase, putative	3.656392	0.0007
74	AT4G33420		peroxidase, putative	3.034868	0.00632
75	AT3G14610	CYP72A7	electron carrier/ heme binding / monooxygenase/ oxygen binding	2.947295	0.000171
76	AT5G14780	FDH	FORMATE DEHYDROGENASE; NAD or NADH binding / binding / catalytic/ cofactor binding / oxidoreductase	2.785644	0.012996
77	AT3G14620	CYP72A8	electron carrier/ heme binding / monooxygenase/ oxygen binding	2.677795	0.00037
78	AT5G43450		2-oxoglutarate-dependent dioxygenase, putative	2.623955	0.000481
79	AT3G13450	DIN4	DARK INDUCIBLE 4; 3-methyl-2-oxobutanoate dehydrogenase	2.562279	0.007083
80	AT4G37410	CYP81F4	electron carrier/ heme binding / monooxygenase/ oxygen binding	2.516202	0.000108
81	AT4G12290; AT4G12280		copper amine oxidase family protein	2.404675	0.049043
82	AT5G50130		short-chain dehydrogenase/reductase (SDR) family protein	2.205913	9.07E-05
83	AT3G20110	CYP705A20	electron carrier/ heme binding / monooxygenase/ oxygen binding	2.159889	0.019475
84	AT2G45570	CYP76C2	electron carrier/ heme binding / monooxygenase/ oxygen binding	2.151626	0.01489
85	AT3G26290	CYP71B26	electron carrier/ heme binding / monooxygenase/ oxygen binding	2.102462	0.001483
86	AT1G23800	ALDH2B7	3-chloroallyl aldehyde dehydrogenase	2.08388	0.015458
87	AT4G37980	ELI3-1, CAD7	ELICITOR-ACTIVATED GENE 3-1; oxidoreductase/ zinc ion binding	2.076803	0.002029
88	AT5G64110		peroxidase, putative	2.035728	0.000999
89	AT1G31710		copper amine oxidase, putative	2.006096	0.03852
Peroxisome					
90	AT3G09260	PYK10, PSR3.1, BGLU23	beta-glucosidase / fucosidase/ hydrolase	14.52884	0.005794
91	AT1G26930		kelch repeat-containing F-box family protein	5.139911	0.000564
92	AT3G15950	NAI2	ER body organization, response to salt stress	3.99937	0.004366

93	AT4G18360	GOX3	GLYCOLATE OXIDASE 3, peroxisomal	3.507086	2.26E-06
94	AT1G52410	TSA1	TSK-ASSOCIATING PROTEIN 1; calcium ion binding	2.584917	0.004693
95	AT5G27600	LACS7	LONG-CHAIN ACYL-COA SYNTHETASE 7	2.35029	0.000182
Response to chemical stimulus					
96	AT2G45210		auxin-responsive protein-related	11.79464	1.19E-05
97	AT4G33540		metallo-beta-lactamase family protein	7.213347	0.000347
98	AT4G18010	IP5PII, AT5PTASE2	MYO-INOSITOL POLYPHOSPHATE 5-PHOSPHATASE 2	4.391116	5.69E-05
99	AT2G41380		embryo-abundant protein-related	4.137567	0.000309
100	AT3G63210	MARD1	MEDIATOR OF ABA-REGULATED DORMANCY 1	4.117897	0.000665
101	AT4G23060	IQD22	IQ-domain 22; calmodulin binding	3.355644	0.000124
102	AT1G28290	AGP31	ARABINOGALACTAN-PROTEIN 31	2.895593	0.047104
103	AT1G07610	MT1C	copper ion binding	2.527668	0.012588
104	AT3G48990	AAE3	ACYL-ACTIVATING ENZYME 3, oxalate degradation	2.246423	0.006626
105	AT1G09570	PHYA, FHY2, FRE1, HY8	PHYTOCHROME A; G-protein coupled photoreceptor	2.064945	0.023521
Response to stress					
106	AT4G23680		major latex protein-related	23.64447	9.69E-05
107	AT3G50970	LTI30, XERO2	LOW TEMPERATURE-INDUCED 30	15.20646	0.000104
108	AT4G23600	CORI3	CORONATINE INDUCED 1; cystathionine beta-lyase/ transaminase	7.4803	8.93E-07
109	AT5G59320	LTP3	LIPID TRANSFER PROTEIN 3	7.354773	0.022232
110	AT4G35770	SEN1, DIN1	SENESCENCE 1	6.780057	0.057303
111	AT3G62550		universal stress protein (USP) family protein	6.312355	0.029628
112	AT5G59310	LTP4	LIPID TRANSFER PROTEIN 4	5.709177	0.002963
113	AT2G03760	RAR047, ST1	brassinosteroid sulfotransferase	5.253201	0.000687
114	AT1G72900		disease resistance protein (TIR-NBS class), putative	5.037367	0.005756
115	AT3G16450		jacalin lectin family protein	4.886648	0.002338
116	AT4G37990	ELI3-2, ATCAD8, CAD-B2	ELICITOR-ACTIVATED GENE 3-2; aryl-alcohol dehydrogenase/ mannitol dehydrogenase	4.832402	0.012029
117	AT2G18050	HIS1-3	HISTONE H1-3; DNA binding	4.374517	0.042773
118	AT3G02480		ABA-responsive protein-related	4.333186	0.008918
119	AT5G22300	NIT4	NITRILASE 4; 3-cyanoalanine hydratase/ indole-3-acetonitrile nitrilase	4.251954	0.009543

120	AT1G29395	COR414-TM1	COLD REGULATED 414 THYLAKOID MEMBRANE 1, cellular response to water deprivation	4.043442	0.002945
121	AT2G33380	RD20	RESPONSIVE TO DESSICATION 20; calcium ion binding	3.752382	0.01044
122	AT2G21620	RD2	response to desiccation, galactolipid biosynthetic process	3.674696	0.000442
123	AT1G18020; AT1G17990		12-oxophytodienoate reductase, putative	3.262797	0.026082
124	AT1G20440	COR47, RD17	COLD-REGULATED 47	3.201476	0.000524
125	AT5G28010		Bet v I allergen family protein	3.078546	0.000808
126	AT3G16460		jacalin lectin family protein	3.006419	0.023107
127	AT4G37530; AT4G37520	at4g37520:PER50, PRXR2	at4g37530: peroxidase, putative at4g37520: peroxidase 50	2.956057	0.010932
128	AT2G18170	ATMPK7	ARABIDOPSIS THALIANA MAP KINASE 7	2.92323	0.016671
129	AT1G12780	UGE1	UDP-D-glucose/UDP-D-galactose 4-epimerase 1	2.304708	0.020594
130	AT3G05360	AtRLP30	Receptor Like Protein 30	2.301642	0.021987
131	AT3G16470	JR1	JASMONATE RESPONSIVE 1	2.269684	0.004786
132	AT4G33950	SRK2E, SNRK2.6, OST1	OPEN STOMATA 1; calcium-dependent protein serine/threonine kinase	2.261583	0.00071
133	AT2G21110		disease resistance-responsive family protein	2.25472	0.017517
134	AT3G17790	ATACP5, PAP17	acid phosphatase	2.227106	0.007559
135	AT1G52000		jacalin lectin family protein	2.201031	0.036732
136	AT1G52030; AT1G52040	at1g52040: MBP1 at1g52030: MBP2	at1g52040: MYROSINASE-BINDING PROTEIN 1 at1g52030:MYROSINASE-BINDING PROTEIN 2; thioglucosidase binding	2.195005	0.040268
137	AT1G74020	SS2	STRICTOSIDINE SYNTHASE 2	2.108054	0.028429
138	AT1G70830	MLP28	MLP-LIKE PROTEIN 28	2.101261	0.018812
139	AT4G24220	VEP1	VEIN PATTERNING 1	2.086551	0.002036
140	AT5G46180	DELTA-OAT	ornithine-oxo-acid transaminase	2.049495	0.00153
Secondary metabolic process					
141	AT3G13610		2OG-Fe(II) oxygenase family protein	5.134753	0.012592
142	AT1G72680	ATCAD1	cinnamyl-alcohol dehydrogenase, putative	8.793325	0.00054
143	AT2G29460	ATGSTU4, GST22	ARABIDOPSIS THALIANA GLUTATHIONE S-TRANSFERASE TAU 4	5.629217	0.000318
144	AT5G48180	NSP5	NITRILE SPECIFIER PROTEIN 5	5.026365	2.62E-06
145	AT1G09500		cinnamyl-alcohol dehydrogenase family	4.804379	0.040677
146	AT2G02990	RNS1	RIBONUCLEASE 1	4.127582	0.019477
147	AT2G02390	ATGSTZ1, GST18	ARABIDOPSIS THALIANA GLUTATHIONE S-	3.996039	0.001144

			TRANSFERASE ZETA 1; glutathione transferase		
148	AT5G25900	GA3, CYP701A3, ATKO1	GA REQUIRING 3; ent-kaurene oxidase	3.435863	0.001871
149	AT1G17170	ATGSTU24	GLUTATHIONE S-TRANSFERASE TAU 24	2.862503	0.000643
150	AT3G57010		strictosidine synthase family protein	2.571519	0.000279
151	AT2G33590		cinnamoyl-CoA reductase family	2.480315	0.000322
152	AT2G25450		2-oxoglutarate-dependent dioxygenase, putative	2.466493	0.000827
153	AT3G16410; AT3G16390; AT3G16400	at3g16410: NSP4 at3g16390: NSP3 at3g16400: NSP1	NITRILE SPECIFIER PROTEIN	2.309807	0.019399
154	AT1G59700	ATGSTU16	GLUTATHIONE S-TRANSFERASE TAU 16	2.272223	9.27E-05
Transcription factor					
155	AT1G77450	ANAC032	Arabidopsis NAC domain containing protein 32	115.4942	4.61E-09
156	AT1G10585		bHLH-type transcription factor	43.08498	1.48E-06
157	AT5G10140	FLC, FLF, AGL25	FLOWERING LOCUS C; specific transcriptional repressor/ transcription factor	11.57218	0.000308
158	AT5G13330	Rap2.6L	RELATED to AP2 6L	7.978512	0.000715
159	AT4G34590; AT4G34588	at4g34590: GBF6, AtbZIP11 at4g34588: CPuORF2	at4g34590: G-BOX BINDING FACTOR 6 at4g34588: Conserved peptide upstream open reading frame 2	6.264385	0.001517
160	AT3G04070	ANAC047	Arabidopsis NAC domain containing protein 47	5.608168	0.013506
161	AT4G18170	WRKY28	response to cyclopentenone, toxin catabolic process	4.432704	1.53E-06
162	AT3G29035	ANAC059,ATNAC3	ARABIDOPSIS NAC DOMAIN CONTAINING PROTEIN 3	4.009915	0.00449
163	AT5G39610	ATNAC2, ORE1, ANAC092, ATNAC6	ARABIDOPSIS NAC DOMAIN CONTAINING PROTEIN 6	3.812219	0.017287
164	AT5G24800	BZIP9	BASIC LEUCINE ZIPPER 9	3.721028	0.000381
165	AT5G49450; AT5G49448	at5g49450: AtbZIP1 at5g49448: CPuORF4	at5g49450: Arabidopsis thaliana basic leucine-zipper 1 at5g49448: Conserved peptide upstream open reading frame 4	3.532008	0.034164
166	AT1G06180	ATMYB13	MYB DOMAIN PROTEIN 13	3.43887	0.000615
167	AT2G36080		DNA-binding protein, putative	3.259187	0.000885
168	AT5G67110	ALC	ALCATRAZ	2.925724	0.030792
169	AT3G04670	WRKY39	calmodulin binding / transcription factor	2.902888	9.35E-06
170	AT5G50915		basic helix-loop-helix (bHLH) family protein	2.739857	0.018584

171	AT1G52890	ANAC019	Arabidopsis NAC domain containing protein 19	2.554885	0.039508
172	AT4G36930	SPT	SPATULA, negative regulation of seed germination	2.476109	0.002213
173	AT5G01200		myb family transcription factor	2.4257	0.001789
174	AT2G42280		basic helix-loop-helix (bHLH) family protein	2.342044	4.87E-05
175	AT1G01010	ANAC001	Arabidopsis NAC domain containing protein 1	2.251101	0.000809
176	AT3G57800		basic helix-loop-helix (bHLH) family protein	2.131992	5.55E-05
177	AT5G65640	BHLH093	beta HLH protein 93	2.063423	0.004762
178	AT5G13180	ANAC083	ARABIDOPSIS NAC DOMAIN CONTAINING PROTEIN 83	2.049529	0.00015
Transporter					
179	AT1G66570	ATSUC7	Sucrose-proton symporter 7;	24.81859	0.000148
180	AT2G04040	ATDTX1, TX1	multidrug efflux pump	14.20818	0.000732
181	AT5G13490	AAC2	ADP/ATP carrier 2; ATP:ADP antiporter	10.32981	2.92E-06
182	AT2G17500		auxin efflux carrier family protein	9.431891	4.26E-06
183	AT5G17860	CAX7	calcium exchanger 7; calcium:sodium antiporter	6.942018	2.77E-05
184	AT3G59140	ATMRP14	ATPase, coupled to transmembrane movement of substances	6.93742	0.001604
185	AT3G60970; AT3G60160	at3g60970: ATMRP15 at3g60160: ATMRP9	ATPase, coupled to transmembrane movement of substances	6.129323	1.79E-05
186	AT1G08230		gamma-aminobutyric acid transmembrane transporter activity	6.072167	2.00E-05
187	AT3G23560	ALF5	ABERRANT LATERAL ROOT FORMATION 5; drug transporter	5.590587	0.000172
188	AT1G71140		MATE efflux family protein	5.231011	0.001079
189	AT3G23550		MATE efflux family protein	3.633738	0.02648
190	AT1G30840	ATPUP4	purine transmembrane transporter	3.44765	0.000305
191	AT3G21690		MATE efflux family protein	3.134244	0.000123
192	AT1G61890		MATE efflux family protein	3.074746	0.000923
193	AT5G13750	ZIFL1	ZINC INDUCED FACILITATOR-like 1; tetracycline:hydrogen antiporter	3.073315	1.52E-05
194	AT2G38290	ATAMT2	AMMONIUM TRANSPORTER 2;	2.779288	6.18E-05
195	AT2G47800	ATMRP4	ARABIDOPSIS THALIANA MULTIDRUG RESISTANCE-ASSOCIATED PROTEIN 4; folic acid transporter	2.728204	0.016759
196	AT3G55130	ATWBC19	White-Brown Complex homolog 19; ATPase, coupled to transmembrane movement of substances	2.573647	0.000974

197	AT1G25530		lysine and histidine specific transporter, putative	2.497767	0.000126
198	AT2G48020		sugar transporter, putative	2.419541	0.000772
199	AT3G20660	AtOCT4	Arabidopsis thaliana ORGANIC CATION/CARNITINE TRANSPORTER4; sugar:hydrogen symporter	2.405715	4.14E-05
200	AT2G23150	NRAMP3	NATURAL RESISTANCE-ASSOCIATED MACROPHAGE PROTEIN 3; inorganic anion transmembrane transporter	2.385905	0.002785
201	AT5G57090	EIR1, WAV6, PIN2, AGR1	ETHYLENE INSENSITIVE ROOT); auxin efflux transmembrane transporter	2.364162	0.000354
202	AT1G74810	BOR5	anion exchanger	2.341599	0.015416
203	AT5G15240		amino acid transporter family protein	2.330684	0.004158
204	AT1G33110		MATE efflux family protein	2.204309	0.001396
205	AT1G66760		MATE efflux family protein	2.198126	0.00478
206	AT1G58360	AAP1, NAT2	AMINO ACID PERMEASE 1; neutral amino acid transmembrane transporter	2.06958	0.002763
Other functions					
207	AT3G60140	DIN2, BGLU30	DARK INDUCIBLE 2; hydrolase	5.933911	0.022664
208	AT2G23810	TET8	TETRASPANIN8	2.239496	0.014333
209	AT1G69880	ATH8	thioredoxin H-type 8	25.1422	0.000349
210	AT1G02850	BGLU11	BETA GLUCOSIDASE 11; hydrolase	19.66124	2.18E-06
211	AT1G67810	SUFE2	SULFUR E 2; enzyme activator	9.767296	0.00326
212	AT4G22530		embryo-abundant protein-related	9.691002	9.94E-06
213	AT2G39310	JAL22	JACALIN-RELATED LECTIN 22	8.906152	0.000419
214	AT5G62110		DNA binding	7.284636	0.0004
215	AT4G22490		protease inhibitor/lipid transfer protein (LTP) family protein	7.247641	0.011454
216	AT3G28007	SWEET4	nodulin MtN3 family protein	6.922294	0.000184
217	AT5G38530		tryptophan synthase-related	6.685648	5.52E-05
218	AT1G09240	NAS3	NICOTIANAMINE SYNTHASE 3	6.207736	0.004125
219	AT4G01430	UMAMIT29	nodulin MtN21 family protein	6.067275	0.000352
220	AT4G28040	UMAMIT33	nodulin MtN21 family protein	5.511548	2.13E-05
221	AT5G17380		pyruvate decarboxylase family protein	5.47592	6.20E-05
222	AT5G54840	SGP1	GTP binding	5.199143	2.76E-06
223	AT5G16080	AtCXE17	Arabidopsis thaliana carboxyesterase 17	4.549698	0.002053
224	AT4G31860		protein phosphatase 2C, putative	4.533403	0.000599
225	AT2G42840	PDF1	PROTODERMAL FACTOR 1	4.445051	0.038263

226	AT4G22470		protease inhibitor/ lipid transfer protein (LTP) family protein	4.33971	0.0211
227	AT3G16430; AT3G16420	at3g16430: JAL31 at3g16420:PBP1, JAL30	at3g16430: JACALIN-RELATED LECTIN 31; copper ion binding at3g16420: PYK10-BINDING PROTEIN 1	4.299153	0.004253
228	AT5G47060		senescence-associated protein-related	4.254011	0.002179
229	AT5G59510	RTFL5, DVL18	ROTUNDIFOLIA LIKE 5	4.19628	0.003787
230	AT1G63840		zinc finger (C3HC4-type RING finger) family protein	4.149747	2.08E-05
231	AT4G27260	GH3.5, WES1	indole-3-acetic acid amido synthetase	4.027045	0.000746
232	AT2G37870		protease inhibitor/ lipid transfer protein (LTP) family protein	3.746455	0.000342
233	AT5G65660		hydroxyproline-rich glycoprotein family protein	3.715762	0.000399
234	AT4G14690	ELIP2	EARLY LIGHT-INDUCIBLE PROTEIN 2; chlorophyll binding	3.61221	0.003698
235	AT5G65390	AGP7	arabinogalactan protein 7 sterol biosynthetic process	3.465446	0.000903
236	AT1G15670		kelch repeat-containing F-box family protein	3.450734	0.029469
237	AT2G39330	JAL23	JACALIN-RELATED LECTIN 23	3.411523	0.00193
238	AT3G19910		zinc finger (C3HC4-type RING finger) family protein	3.375936	0.001933
239	AT1G61330; AT1G61320		at1g61330: F-box family protein at1g61320: unknown	3.367776	0.039321
240	AT2G02710	PLPB	PAS/LOV PROTEIN B; two-component sensor	3.255754	0.02996
241	AT3G10450	SCPL7	SERINE CARBOXYPEPTIDASE-LIKE 7	3.158142	0.010542
242	AT1G66480	PMI2	plastid movement impaired 2	3.131384	2.76E-05
243	AT1G23440		pyrrolidone-carboxylate peptidase family protein	3.017379	0.000161
244	AT4G01870		tolB protein-related	2.971778	0.019122
245	AT5G54510	GH3.6, DFL1	DWARF IN LIGHT 1; indole-3-acetic acid amido synthetase	2.944273	0.018896
246	AT1G31880; AT2G21030	at1g31880: NLM9, BRX	at1g31880: BREVIS RADIX; water channel at2g21030	2.941479	0.018305
247	AT1G76790		O-methyltransferase family 2 protein	2.941472	0.000301
248	AT1G52690		late embryogenesis abundant protein, putative	2.922277	0.038053
249	AT2G16700	ADF5	ACTIN DEPOLYMERIZING FACTOR 5	2.832693	0.001892
250	AT5G37740		C2 domain-containing protein	2.775706	0.009582
251	AT3G08860		alanine--glyoxylate aminotransferase, putative	2.760281	0.002744
252	AT1G28130	GH3.17	indole-3-acetic acid amido synthetase	2.75818	1.39E-05
253	AT1G04990		zinc finger (CCCH-type) family protein	2.721602	0.00903

254	AT2G37080		myosin heavy chain-related	2.695395	0.001345
255	AT4G24040	TRE1	TREHALASE 1	2.669742	0.004006
256	AT2G30400	OFP2	OVATE FAMILY PROTEIN 2	2.640737	0.005312
257	AT5G01210		transferase family protein	2.634845	0.015089
258	AT5G16450		dimethylmenaquinone methyltransferase family protein	2.616874	0.000145
259	AT1G32940	SBT3.5	serine-type endopeptidase	2.60018	0.048905
260	AT4G25835		AAA-type ATPase family protein	2.582127	0.001425
261	AT2G04160	AIR3	serine-type endopeptidase	2.581508	0.004246
262	AT3G17820	ATGSKB6, GLN1.3	glutamate-ammonia ligase	2.578566	0.000295
263	AT4G19420		pectinacetylesterase family protein	2.551852	5.86E-05
264	AT1G22500		zinc finger (C3HC4-type RING finger) family protein	2.48845	0.03545
265	AT3G53210	UMAMIT6	nodulin MtN21 family protein	2.438857	0.001289
266	AT2G28120		nodulin family protein	2.393495	0.029096
267	AT5G24090	CHIA	acidic endochitinase	2.388647	0.005299
268	AT2G28320		pleckstrin homology (PH) domain-containing protein	2.371697	0.001786
269	AT1G70790		C2 domain-containing protein	2.347914	0.000306
270	AT1G74790		Catalytic acting on CH-OH group of donors	2.333346	0.000108
271	AT1G66180		aspartyl protease family protein	2.33087	0.003705
272	AT1G58889; AT1G59265		transposable element gene	2.327822	0.044887
273	AT5G23820		MD-2-related lipid recognition domain-containing protein	2.321514	0.00735
274	AT3G04010		glycosyl hydrolase family 17 protein	2.281267	0.023084
275	AT3G03440		armadillo/beta-catenin repeat family protein	2.254747	0.008427
276	AT4G36760	ATAPP1	N-1-naphthylphthalamic acid binding	2.209365	0.001041
277	AT1G30820		CTP synthase, putative	2.183089	0.006337
278	AT2G27200		GTP-binding family protein	2.181652	0.032579
279	AT4G16250	PHYD	PHYTOCHROME DEFECTIVE D; G-protein coupled photoreceptor	2.180387	2.75E-05
280	AT1G70782; AT1G70780	at1g70782:CPuORF28	at1g70782: Conserved peptide upstream open reading frame 28 at1g70780: unknown protein	2.151836	0.001318
281	AT1G55500	ECT4	EVOLUTIONARILY CONSERVED C-TERMINAL REGION 4	2.142107	0.038379
282	AT1G44800		nodulin MtN21 family protein	2.1393	0.00494
283	AT1G43590		transposable element gene	2.11851	0.003507
284	AT1G30860		protein binding / zinc ion binding	2.114061	0.002425
285	AT4G16540;		heat shock protein-related	2.113697	0.019747

	AT2G03020				
286	AT3G54950	PLP7, PLA IIIA	PATATIN-LIKE PROTEIN 6	2.10234	0.000386
287	AT3G29810	COBL2	COBRA-LIKE PROTEIN 2 PRECURSOR	2.101852	0.01046
288	AT4G31590	ATCSLC5	CELLULOSE-SYNTHASE LIKE C5	2.093423	0.004987
289	AT2G06255	ELF4-L3	ELF4-Like 3	2.087513	0.000738
290	AT2G36400	AtGRF3	GROWTH-REGULATING FACTOR 3	2.086138	0.016282
291	AT1G01070	UMAMIT28	nodulin MtN21 family protein	2.064515	0.035635
292	AT4G21510		F-box family protein	2.062487	0.022613
293	AT1G63010		SPX (SYG1/Pho81/XPR1) domain-containing protein	2.037807	0.000255
294	AT3G10190		calmodulin, putative	2.025874	0.001429
295	AT1G52080	AR791	actin binding	2.022744	0.003086
296	AT3G53980		protease inhibitor/ lipid transfer protein (LTP) family protein	2.021752	0.02841
297	AT3G02740		aspartyl protease family protein	2.01532	0.000655
298	AT3G26690	ATNUDX13	ARABIDOPSIS THALIANA NUDIX HYDROLASE HOMOLOG 13	29.74907	3.83E-08
299	AT1G34040; AT1G34060		alliinase family protein	13.44782	1.23E-05
300	AT3G10912; AT3G10910	at3g10912: CPuORF63	at3g10912: Conserved peptide upstream open reading frame 63 at3g10910: zinc finger (C3HC4-type RING finger) family protein	11.50478	1.89E-06
301	AT5G39050		transferring acyl groups other than amino-acyl groups	10.47903	3.81E-05
302	AT1G67820		protein phosphatase 2C, putative	8.569184	0.000124
303	AT5G22860		serine carboxypeptidase S28 family protein	8.229937	0.000128
304	AT5G13370; AT5G13360		auxin-responsive GH3 family protein	7.306631	2.45E-05
305	AT4G24000	CSLG2	cellulose synthase/ transferring glycosyl groups	6.028111	0.001352
306	AT5G41070	DRB5	DSRNA-BINDING PROTEIN 5	5.179459	0.000115
307	AT4G27830	BGLU10	BETA GLUCOSIDASE 10; hydrolase	4.506837	0.000157
308	AT1G69870		proton-dependent oligopeptide transport (POT) family protein	3.488548	0.000244
309	AT5G67480	BT4	BTB AND TAZ DOMAIN PROTEIN 4; protein binding / transcription regulator	2.752251	0.021153
310	AT3G10870	MES17	METHYL ESTERASE 17	2.59567	0.010281
311	AT1G78670	ATGGH3	gamma-glutamyl hydrolase 3; omega peptidase	2.507317	0.000186
312	AT5G63140	ATPAP29	ARABIDOPSIS THALIANA PURPLE ACID PHOSPHATASE 29	2.39799	0.002376
313	AT3G14000	ATBRXL2	BRX-LIKE2	2.229507	0.000179

Unknown					
314	AT1G29195		unknown protein	9.785882	5.79E-06
315	AT5G57785		response to sucrose stimulus	8.70065	0.000492
316	AT5G39520		unknown protein	7.472651	0.001497
317	AT3G20300		hyperosmotic salinity response, response to ABA, cold and water deprivation	6.684753	1.62E-07
318	AT5G18130		response to bacteria	6.311842	0.000691
319	AT5G67600		Involved in megasporogenesis	5.295142	1.51E-06
320	AT1G15010		response to fungus	5.087402	0.001343
321	AT1G62045		unknown protein	3.655559	0.00033
322	AT1G05340		response to chitin	3.602973	0.035809
323	AT5G44570		unknown protein	3.595589	0.001261
324	AT2G37750		unknown protein	3.409527	0.002835
325	AT1G27030		unknown protein	3.267671	0.000779
326	AT5G57910		response to phosphate starvation and water deprivation	3.065675	0.008321
327	AT3G48550		unknown protein	3.01456	0.002172
328	AT1G69760		unknown protein	3.0009	0.000304
329	AT1G53870; AT1G53890		unknown protein	2.775956	0.004817
330	AT2G28400		unknown protein	2.76355	0.01528
331	AT1G07040		unknown protein	2.727967	0.006837
332	AT2G24100	ASG1	ALTERED SEED GERMINATION 1	2.722175	0.000111
333	AT4G16000		Unknown protein	2.698386	0.021416
334	AT1G69160		unknown protein	2.609614	0.00075
335	AT5G11070		response to brassinosteroid	2.522679	0.005376
336	AT1G02470		lipid transport superfamily protein	2.429294	0.025678
337	AT2G34070	TBL37	TRICHOME BIREFRINGENCE-LIKE 37	2.365471	0.006245
338	AT3G10120		unknown protein	2.337595	0.020093
339	AT3G19200		unknown protein	2.276176	0.002032
340	AT3G27770		unknown protein	2.217311	0.005692
341	AT1G28190		response to phytohormones ABA,JA,SA and ET	2.215052	0.001976
342	AT5G51200		EMBRYO DEFECTIVE 3142	2.213379	0.000281
343	AT1G78170		unknown protein	2.21131	0.000428
344	AT1G21010		involved in N-terminal protein myristoylation	2.203696	0.025865
345	AT5G43180		unknown protein	2.095066	0.008129
346	AT1G02816		unknown protein	2.07257	0.00029
347	AT5G12420		O-acyltransferase (WSD1-like) family protein	2.054012	0.00496

Table S2: List of genes down-regulated in *35S:ANAC032* plants (>2-fold; P<0.05)

No.	Gene code	Symbol	Description	*Col-0/ 35S:ANAC032* (fold suppression)	p-value
Cell wall related					
1	AT5G03350		legume lectin family protein	3.765392	0.027743
2	AT3G16530		legume lectin family protein E5	3.107994	0.036902
3	AT1G65870		disease resistance-responsive family protein	3.079823	0.032463
4	AT1G04040		acid phosphatase class B family protein	2.866842	0.005916
5	AT3G22060		receptor protein kinase-related	2.401016	0.012702
6	AT2G39850		identical protein binding /serine-type endopeptidase	2.400638	0.001758
7	AT4G37800		xyloglucan:xyloglucosyl transferase, putative	2.370646	0.003114
8	AT3G15400	ATA20	glycine-rich cell wall protein	2.208898	9.50E-05
9	AT2G26440		pectinesterase family protein	2.11728	0.014366
10	AT4G01700		chitinase, putative	2.107568	0.005514
11	AT5G53370	PMEPCRF	PECTIN METHYLESTERASE PCR FRAGMENT F; pectinesterase	2.050275	0.003018
12	AT5G65730		xyloglucan:xyloglucosyl transferase, putative	2.298604	0.034813
Endomembrane system					
13	AT3G47540		chitinase, putative	2.053141	7.30E-05
14	AT2G35860	FLA16	FASCICLIN-LIKE ARABINOGALACTAN PROTEIN 16 PRECURSOR	2.034319	0.03553
15	AT5G50800	SWEET13	nodulin MtN3 family protein	3.140465	0.021547
16	AT3G52720	ACA1	ALPHA CARBONIC ANHYDRASE 1; carbonate dehydratase	3.105561	0.021474
17	AT5G48540		33 kDa secretory protein-related	2.608235	0.000488
18	AT3G48460		GDSL-motif lipase/hydrolase family protein	2.489515	0.043871
19	AT1G19940	AtGH9B5	Arabidopsis thaliana Glycosyl Hydrolase 9B5; hydrolase	2.272411	0.004566
20	AT3G52470		harpin-induced family protein	2.240173	0.000395
21	AT3G29030	ATEXPA5, EXPA5	EXPANSIN A5, plant-type cell wall loosening	2.23436	0.01626
22	AT1G78450		SOUL heme-binding family protein	2.175728	0.002193
23	AT3G51450		strictosidine synthase family protein	2.161901	0.005171
24	AT3G57630		exostosin family protein	2.071314	0.009523
Hydrolase					
25	AT5G58310	MES18	METHYL ESTERASE 18; hydrolase/ methyl indole-3-	4.406805	0.006055

			acetate esterase		
26	AT4G12830		hydrolase, alpha/beta fold family	2.261393	0.041191
Kinase activity					
27	AT4G23130	CRK5, RLK6	CYSTEINE-RICH RLK5; kinase	2.538395	0.011511
28	AT1G21270	WAK2	ATP binding / calcium ion binding / protein kinase	2.013779	0.045028
29	AT5G59670		leucine-rich repeat protein kinase, putative	6.372129	0.00176
30	AT5G60900	RLK1	RECEPTOR-LIKE PROTEIN KINASE 1; ATP binding / kinase	4.514457	0.00819
31	AT5G59680		leucine-rich repeat protein kinase, putative	3.934956	0.000206
32	AT3G45860		receptor-like protein kinase, putative	3.650749	0.009314
33	AT1G29720		protein kinase family protein	2.99631	0.001562
34	AT4G21380	ARK3	A. THALIANA RECEPTOR KINASE 3 ; transmembrane receptor protein serine/threonine kinase	2.599056	0.00043
35	AT1G11330		S-locus lectin protein kinase family protein	2.597267	0.001168
36	AT4G23320		protein kinase family protein	2.57797	0.000182
37	AT4G23260		ATP binding / protein kinase	2.431219	0.016496
38	AT1G78290		serine/threonine protein kinase, putative	2.279841	0.001206
39	AT4G02420		lectin protein kinase, putative	2.222469	0.037288
40	AT1G69730		protein kinase family protein	2.20671	0.001366
41	AT1G51790		kinase	2.175165	0.00227
42	AT1G16260		protein kinase family protein	2.174017	0.000115
43	AT2G23770		protein kinase family protein / peptidoglycan-binding LysM domain-containing protein	2.146823	0.000198
44	AT2G37710	RLK	RECEPTOR LECTIN KINASE	2.117521	0.019708
45	AT5G38990;AT5G39000		protein kinase family protein	2.026729	0.000216
Methyltransferase activity					
46	AT3G44860;AT3G44870	at3g44860: FAMT	FAMT (farnesoic acid carboxyl-O-methyltransferase) at3g44870: S-adenosyl-L-methionine:carboxyl methyltransferase family protein	3.684674	5.71E-05
47	AT1G73602;AT1G73600	at1g73602: CPuORF32	Conserved peptide upstream open reading frame 32 at1g73600: phosphoethanolamine N-methyltransferase	3.010881	0.000382
Oxygen binding					
48	AT2G30770	CYP71A13	indoleacetaldoxime dehydratase	3.602751	0.0224

49	AT5G52320	CYP96A4	heme binding / monooxygenase	2.935683	0.004136
50	AT3G26230	CYP71B24	iron ion binding / monooxygenase	2.306404	0.000714
51	AT3G26320	CYP71B36	iron ion binding / monooxygenase	2.242609	0.002329
52	AT1G13080	CYP71B2	iron ion binding / monooxygenase	2.240076	0.024186
53	AT2G26170	CYP711A1	iron ion binding / monooxygenase	2.102305	0.020876
54	AT5G36220	CYP81D1	iron ion binding / monooxygenase	2.023514	0.043273
Response to stress					
55	AT4G14400	ACD6	ACCELERATED CELL DEATH 6; protein binding	4.38331	0.014703
56	AT2G14560	LURP1	LATE UPREGULATED IN RESPONSE TO HYALOPERONOSPORA PARASITICA	4.220421	0.012204
57	AT1G19670	ATCLH1, CORI1	A.THALIANA CORONATINE-INDUCED PROTEIN 1; chlorophyllase	3.455888	4.54E-05
58	AT4G17090	CT-BMY, BMY8, BAM3	CHLOROPLAST BETA-AMYLASE ; beta-amylase	3.397184	0.017437
59	AT1G54040	ESP, TASTY	EPITHIOSPECIFIER PROTEIN; enzyme regulator	2.957499	0.000294
60	AT1G76690;AT1G76680	at1g76680: OPR1 at1g76690: OPR2	12-oxophytodienoate reductase |	2.954008	0.002281
61	AT1G17610		disease resistance protein-related	2.431586	8.31E-05
62	AT3G16670		unknown protein	2.426182	0.007134
63	AT4G15440	HPL1, CYP74B2	HYDROPEROXIDE LYASE 1; electron carrier/ heme binding/ monooxygenase	2.376575	0.008187
64	AT4G26090	RPS2	RESISTANT TO P. SYRINGAE 2	2.184328	0.001428
65	AT1G80850		methyladenine glycosylase family protein	2.041256	0.012814
66	AT1G72910;AT1G72930	at1g72930: IR	at1g72910: disease resistance protein (TIR-NBS class), putative at1g72930: TOLL /INTERLEUKIN-1 RECEPTOR-LIKE	2.037226	0.004886
Response to stimulus					
67	AT3G22231	PCC1	PATHOGEN AND CIRCADIAN CONTROLLED 1	5.211186	0.011547
68	AT2G40100	LHCB4.3	light harvesting complex PSII; chlorophyll binding	4.624667	0.004588
69	AT5G54610	ANK	ankyrin; protein binding	4.096957	0.0028
70	AT4G13180		short-chain dehydrogenase/ reductase (SDR) family protein	3.731777	0.000547
71	AT5G55450		protease inhibitor/ lipid transfer protein (LTP) family protein	3.565105	0.013979
72	AT1G52770		phototropic-responsive NPH3 family protein	3.416003	0.000182
73	AT1G78020		Senescence-associated protein-related	2.266408	0.007507

74	AT1G30260		response to cytokinin stimulus	2.262901	0.004016
75	AT1G70700	JAZ9, TIFY7	JASMONATE-ZIM-DOMAIN PROTEIN 9	2.245812	0.048306
76	AT4G17670		senescence-associated protein-related	2.20176	0.001727
77	AT4G36220	FAH1, CYP84A1	FERULIC ACID 5-HYDROXYLASE 1; ferulate 5-hydroxylase	2.1968	0.001008
78	AT1G56150		auxin-responsive family protein	2.151447	0.017227
79	AT2G24540	AFR	ATTENUATED FAR-RED RESPONSE	2.053255	0.014936
80	AT3G21950		Methyltransferase	2.049947	0.028911
81	AT3G62410	CP12-2, CP12	protein binding	2.041587	0.021873
Transcription factor					
82	AT5G63790	ANAC102	ARABIDOPSIS NAC DOMAIN CONTAINING PROTEIN 102	3.780047	0.0051
83	AT2G40750	WRKY54	MAPK cascade, negative regulation of defense response	3.290537	0.00141
84	AT5G53200	TRY	TRIPTYCHON; DNA binding	3.095995	0.034495
85	AT1G75250	ATRL6	ARABIDOPSIS RAD-LIKE 6;	3.09007	0.011857
86	AT3G02380	COL2	constans-like 2; transcription factor/ zinc ion binding	2.702784	0.002375
87	AT5G07580		DNA binding / transcription factor	2.433529	0.006766
88	AT2G21650	MEE3	MATERNAL EFFECT EMBRYO ARREST 3	2.256903	0.030744
89	AT3G15270	SPL5	SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 5	2.129177	0.006874
90	AT3G56400	WRKY70	transcription factor/ transcription repressor	2.091257	0.00114
91	AT1G19510	ATRL5	ARABIDOPSIS RAD-LIKE 5	2.033622	0.015531
Transporter					
92	AT1G64780	AMT1;2 ATAMT1;2	AMMONIUM TRANSPORTER 1;2; transmembrane transporter	3.805951	0.049804
93	AT3G46900	COPT2	copper ion transmembrane transporter/ high affinity copper ion transmembrane transporter	3.161913	0.000458
94	AT1G55910	ZIP11	ZINC TRANSPORTER 11 PRECURSOR; cation transmembrane transporter	2.521367	0.003096
95	AT5G45380	DUR3	sodium:solute symporter family protein	2.394784	0.028397
96	AT4G30110	HMA2	cadmium-transporting ATPasex	2.262226	0.03795
97	AT4G13510	ATAMT1;1	AMMONIUM TRANSPORTER 1;1;	2.219013	0.00107
98	ATMG00410;AT2G07741;ATMG01170;AT2G07699	atmg00410: ATP6, atmg01170: ATP6-2	at2g07699: similar to ATPase subunit 6; atmg00410: ATPase subunit 6 at2g07741: ATPase subunit 6, putative atmg01170:ATPase subunit 6-2	2.213852	0.020876

99	AT1G80760	NIP6;1	NOD26-LIKE INTRINSIC PROTEIN 6;1; boron transporter/ glycerol transmembrane transporter	2.173673	0.011656
100	AT1G32450	NRT1.5	NITRATE TRANSPORTER 1.5	2.127833	0.003079
101	AT1G12110	NRT1.1, CHL1, B-1	nitrate transmembrane transporter	2.043714	0.00117
102	AT2G21540	SFH3	SEC14-LIKE 3; phosphatidylinositol transporter	2.02183	0.00107
Other functions					
103	AT5G24150	SQP1, SQE5	squalene monooxygenase	3.713257	0.045456
104	AT2G32290	BMY5, BAM6	BETA-AMYLASE 6; cation binding	2.484569	0.037811
105	AT1G64400		long-chain-fatty-acid--CoA ligase, putative	2.732873	0.00872
106	AT5G10170	MIPS3	MYO-INOSITOL-1-PHOSTPATE SYNTHASE 3	2.496849	0.011415
107	AT2G41180		sigA-binding protein-related	2.794774	0.003105
108	AT4G03450		ankyrin repeat family protein	2.595286	0.008315
109	AT3G48080		lipase class 3 family protein / disease resistance protein-related	2.459949	0.02599
110	AT3G56710	SIB1	SIGMA FACTOR BINDING PROTEIN 1	2.44967	0.006026
111	AT4G04840	ATMSRB6	methionine sulfoxide reductase domain-containing protein	2.374638	0.000339
112	AT3G57020		strictosidine synthase family protein	2.368128	0.000295
113	AT2G39420		esterase/lipase/thioesterase family protein	2.353474	0.000186
114	AT3G24503	ALDH2C4	3-chloroallyl aldehyde dehydrogenase	2.35122	0.007888
115	AT4G08300	UMAMIT17	nodulin MtN21 family protein	2.350839	0.021292
116	AT1G01390		UDP-glucoronosyl/UDP-glucosyl transferase family protein	2.307895	0.007425
117	AT2G39210		nodulin family protein	2.306359	0.006015
118	AT1G69720	HO3	HEME OXYGENASE 3	2.200228	0.000766
119	AT4G20780		calcium-binding protein, putative	2.196002	9.55E-05
120	AT1G24140		matrixin family protein	2.126652	0.007713
121	AT1G22650		beta-fructofuranosidase, putative / invertase, putative	2.060497	0.0098
122	AT4G22570	APT3	ADENINE PHOSPHORIBOSYL TRANSFERASE 3	2.042801	0.039514
123	AT1G64500		glutaredoxin family protein	2.036543	0.023248
124	AT1G55850	CSLE1	cellulose synthase/ transferring glycosyl groups	2.02917	0.004153
125	AT3G49580	LSU1	RESPONSE TO LOW SULFUR 1	2.024903	0.033663
126	AT2G32880;AT2G32870		meprin and TRAF homology domain-containing protein, MATH domain-containing protein	2.013923	0.001823

127	AT2G47130		short-chain dehydrogenase/reductase (SDR) family protein	2.563469	0.003639
Unknown					
128	AT2G46630		unknown protein	2.002048	0.001041
129	AT1G65490		unknown protein	4.271573	0.005519
130	AT3G22240		unknown protein	3.498544	0.011788
131	AT5G19240		unknown protein	2.966056	0.000808
132	AT5G22390		unknown protein	2.933623	0.000495
133	AT1G19960		unknown protein	2.927	0.040121
134	AT3G51750		unknown protein	2.918411	0.033997
135	AT1G74440		unknown protein	2.900198	0.01844
136	AT2G15020		myo-inositol hexakisphosphate biosynthetic process	2.875841	0.024421
137	AT5G01790		unknown protein	2.854305	0.008125
138	AT1G68600		Aluminium activated malate transporter family protein	2.555495	0.000237
139	AT2G32160		S-adenosyl-L-methionine-dependent methyltransferases superfamily protein	2.46698	0.008123
140	AT1G55960		lipid transport superfamily protein	2.393015	0.047731
141	AT5G35490	MRU1	MTO1-RESPONDING UP 1	2.268254	0.027792
142	AT2G31110	TBL40	TRICHOME BIREFRINGENCE-LIKE 40	2.218512	0.000361
143	AT4G00955		unknown protein	2.19896	0.000711
144	AT5G42860		unknown protein	2.156481	0.005854
145	AT1G11700		unknown protein	2.128422	0.025579
146	AT5G44820		Nucleotide-diphospho-sugar transferase family protein	2.076036	0.042788
147	AT5G01015		unknown protein	2.056488	0.017074

Table S3: List of common genes up-regulated by TIBA and by over-expression of *35S:ANAC032* (>2-fold, P<0.05)

No.	Gene code	Symbol	Description	*35S:ANAC032/ Col-0*	*Col-0* TIBA/mock
1	AT1G77450	ANAC032	NAC domain containing protein 32	115.4942	9.09966
2	AT2G37770	AKR4C9	aldo/keto reductase family protein	56.89307	3.280615
3	AT1G10585		bHLH type transcription factor	43.08498	18.91797
4	AT1G02850	BGLU11	BETA GLUCOSIDASE 11; hydrolase	19.66124	5.033623
5	AT2G43820	UGT74F2	UDP-GLUCOSYLTRANSFERASE 74F2	18.93009	2.791824
6	AT3G04000		short-chain dehydrogenase/ reductase (SDR) family protein	13.73017	5.135259
7	AT2G37760	AKR4C8	aldo/keto reductase family protein	12.63507	3.627012
8	AT4G15490	UGT84A3	UDP-glycosyltransferase/ sinapate 1-glucosyltransferase	12.35113	3.503894
9	AT2G45210		auxin-responsive protein-related	11.79464	2.066764
10	AT5G39050		transferring acyl groups other than amino-acyl groups	10.47903	2.80455
11	AT5G13490	AAC2	ADP/ATP carrier 2	10.32981	2.214483
12	AT1G67810	SUFE2	SULFUR E 2; enzyme activator	9.767296	5.242495
13	AT4G22530		embryo-abundant protein-related	9.691002	2.618454
14	AT1G05680	UGT74E2	UDP-glucosyl transferase family protein	9.512384	20.43049
15	AT2G17500		auxin efflux carrier protein family	9.431891	6.254818
16	AT5G13330	Rap2.6L	related to AP2 6L; transcription factor	7.978512	17.93513
17	AT1G26770	ATEXPA10	ARABIDOPSIS THALIANA EXPANSIN A 10; structural constituent of cell wall	7.724935	2.105974
18	AT3G46660	UGT7E12	UDP-GLUCOSYL TRANSFERASE 76E12	7.649356	2.246742
19	AT4G33540		metallo-beta-lactamase family protein	7.213347	2.04123
20	AT5G17860	CAX7	calcium exchanger 7; calcium:sodium antiporter	6.942018	3.505503
21	AT3G59140	ATMRP14	ATPase, coupled to transmembrane movement of substances	6.93742	2.140471
22	AT4G35770	SEN1	SENESCENCE 1	6.780057	2.129441
23	AT1G76470		3-beta-hydroxy-delta5-steroid dehydrogenase	6.669548	2.284777
24	AT4G34590; AT4G34588	at4g34590: BZIP11 GBF6 at4g34588: CPuORF2	At4g34590: G-BOX BINDING FACTOR 6; transcription factor at4g34588: Conserved peptide upstream open reading frame 2	6.264385	2.045682
25	AT2G29460	ATGSU4	ARABIDOPSIS THALIANA GLUTATHIONE S-	5.629217	6.711621

			TRANSFERASE TAU 4		
26	AT3G04070	ANAC047	Arabidopsis NAC domain containing protein 47	5.608168	2.346065
27	AT2G34810		FAD-binding domain-containing protein	5.385956	2.34858
28	AT2G03760	ST1	brassinosteroid sulfotransferase	5.253201	2.854676
29	AT1G71140		MATE efflux family protein	5.231011	2.105899
30	AT1G72680		oxidoreductase, 2OG-Fe(II) oxygenase family protein	5.134753	2.251101
31	AT1G72900		disease resistance protein (TIR-NBS class), putative	5.037367	4.303723
32	AT3G16450		Jacalin lectin family protein	4.886648	2.194627
33	AT5G16080	AtCXE17	Arabidopsis thaliana carboxyesterase 17	4.549698	3.021722
34	AT4G18010	IP5PII	MYO-INOSITOL POLYPHOSPHATE 5-PHOSPHATASE 2	4.391116	2.468035
35	AT4G22470		protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	4.33971	3.454267
36	AT5G22300	NIT4	NITRILASE 4; 3-cyanoalanine hydratase/ indole-3-acetonitrile nitrilase	4.251954	3.836168
37	AT5G59510	RTFL5 DVL18	ROTUNDIFOLIA LIKE 5	4.19628	2.216006
38	AT1G63840		zinc finger (C3HC4-type RING finger) family protein	4.149747	2.08117
39	AT2G02990	RNS1	RIBONUCLEASE 1	4.127582	2.23622
40	AT4G27260	WES1	indole-3-acetic acid amido synthetase	4.027045	2.259881
41	AT2G30140		UDP-glucoronosyl/UDP-glucosyl transferase family protein	3.745069	2.280057
42	AT5G51830		pfkB-type carbohydrate kinase family protein	3.66412	2.266256
43	AT4G11320; AT4G11310		cysteine proteinase, putative	3.660721	3.876716
44	AT5G06860	PGIP1	POLYGALACTURONASE INHIBITING PROTEIN 1	3.653244	2.665013
45	AT3G23550		MATE efflux family protein	3.633738	9.949475
46	AT1G15670		kelch repeat-containing F-box family protein	3.450734	2.036242
47	AT2G39330	JAL23	JACALIN-RELATED LECTIN 23	3.411523	4.038976
48	AT4G14130	XTR7	XYLOGLUCAN ENDOTRANSGLYCOSYLASE 7	3.401027	3.899549
49	AT1G32960	SBT3.3	identical protein binding / serine-type endopeptidase	3.331092	2.419061
50	AT5G13750	ZIFL1	ZINC INDUCED FACILITATOR-like 1; tetracycline:hydrogen antiporter	3.073315	3.235837
51	AT1G54000; AT1G54010	MPL1	MYZUS PERSICAE-INDUCED LIPASE 1	3.016294	2.961967
52	AT3G16460		Jacalin lectin family protein	3.006419	2.492469
53	AT4G01870		tolB protein-related	2.971778	7.612111
54	AT4G37530;AT4G37520	at4g37520: PER50	at4g37530: peroxidase, putative at4g37520: peroxidase 50	2.956057	3.161199

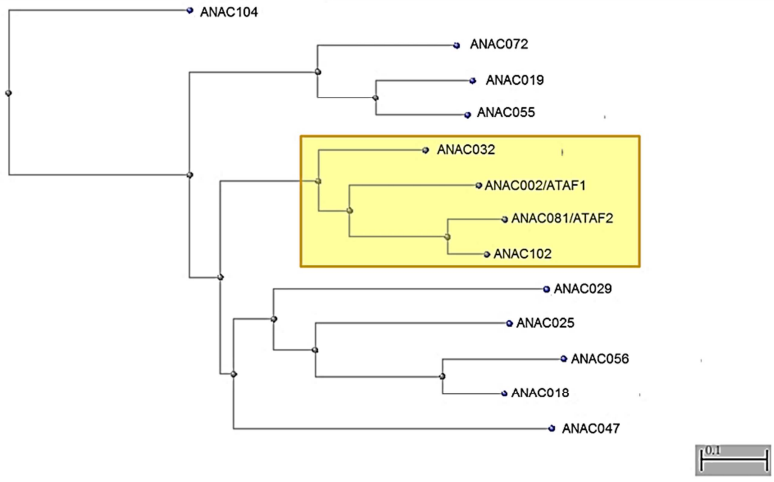
55	AT1G76790		O-methyltransferase family 2 protein	2.941472	3.815661
56	AT1G17170	GSTU24	GLUTATHIONE S-TRANSFERASE TAU 24	2.862503	28.97755
57	AT5G67480	BT4	BTB AND TAZ DOMAIN PROTEIN 4	2.752251	2.369505
58	AT5G50915		basic helix-loop-helix (bHLH) family protein	2.739857	2.025977
59	AT2G24100		unknown protein	2.722175	2.990254
60	AT3G14620	CYP72A8	electron carrier/ heme binding / monooxygenase/ oxygen binding	2.677795	2.137935
61	AT4G24040	TRE1	TREHALASE 1	2.669742	2.104687
62	AT3G55130	ATWBC19	White-Brown Complex homolog 19; coupled to transmembrane movement of substances	2.573647	2.07274
63	AT1G52890	ANAC019	Arabidopsis NAC domain containing protein 19	2.554885	2.966996
64	AT5G07010	ST2A	SULFOTRANSFERASE 2A	2.545133	2.246032
65	AT5G06870	PGIP2	POLYGALACTURONASE INHIBITING PROTEIN 2	2.528479	3.956469
66	AT4G37410	CYP81F4	electron carrier/ heme binding / iron ion binding / monooxygenase/ oxygen binding	2.516202	7.608564
67	AT4G12290; AT4G12280		copper amine oxidase family protein	2.404675	2.090719
68	AT2G02000;AT2G02010	at2g02000: GAD3 at2g02010: GAD4	at2g02000: glutamate decarboxylase 3; at2g02010: glutamate decarboxylase 4)	2.370822	2.607742
69	AT2G34070		unknown protein	2.365471	2.225622
70	AT5G27600	LACS7	LONG-CHAIN ACYL-COA SYNTHETASE 7	2.35029	2.325535
71	AT3G16410; AT3G16390; AT3G16400	at3g16410: NSP4 at3g16390: NSP3 at3g16400: NSP1	NITRILE SPECIFIER PROTEIN	2.309807	2.374808
72	AT3G05360	AtRLP3	Receptor Like Protein 30	2.301642	2.263713
73	AT3G16470	JR1		2.269684	2.125185
74	AT1G05560	UGT75B1	UDP-glycosyltransferase	2.219308	11.38664
75	AT1G33110		MATE efflux family protein	2.204309	3.47544
76	AT1G66760		MATE efflux family protein	2.198126	3.801804
77	AT3G29810	COBL2	COBRA-LIKE PROTEIN 2 PRECURSOR	2.101852	2.230228
78	AT5G12420		unknown protein	2.054012	2.468399

Table S4: List of common genes down-regulated by TIBA and by over-expression of *35S:ANAC032* (>2-fold, P<0.05)

No.	Gene code	Symbol	Description	*35S:ANAC032/ Col-0*	*Col-0* TIBA/mock
1	AT5G59670		LRR protein kinase family protein	6.372129	3.020526
2	AT2G14560	LURP1	LATE UPREGULATED IN RESPONSE TO HYALOPERONOSPORA PARASITICA 1	4.220421	2.165947
3	AT5G03350		legume lectin family protein	3.765392	2.76781
4	AT2G40750	WRKY54	transcription factor	3.290537	2.149989
5	AT3G52720	ACA1	ALPHA CARBONIC ANHYDRASE 1; carbonate dehydrates activity	3.105561	2.672328
6	AT1G19960		unknown	2.927	2.531967
7	AT2G39850		serine type endopeptidase	2.400638	3.060939
8	AT1G80760	NIP6;1, NLM7	NOD26-LIKE INTRINSIC PROTEIN 6;1; boron transporter/ glycerol transmembrane transporter/ urea transmembrane transporter/ water channel	2.173673	2.118307

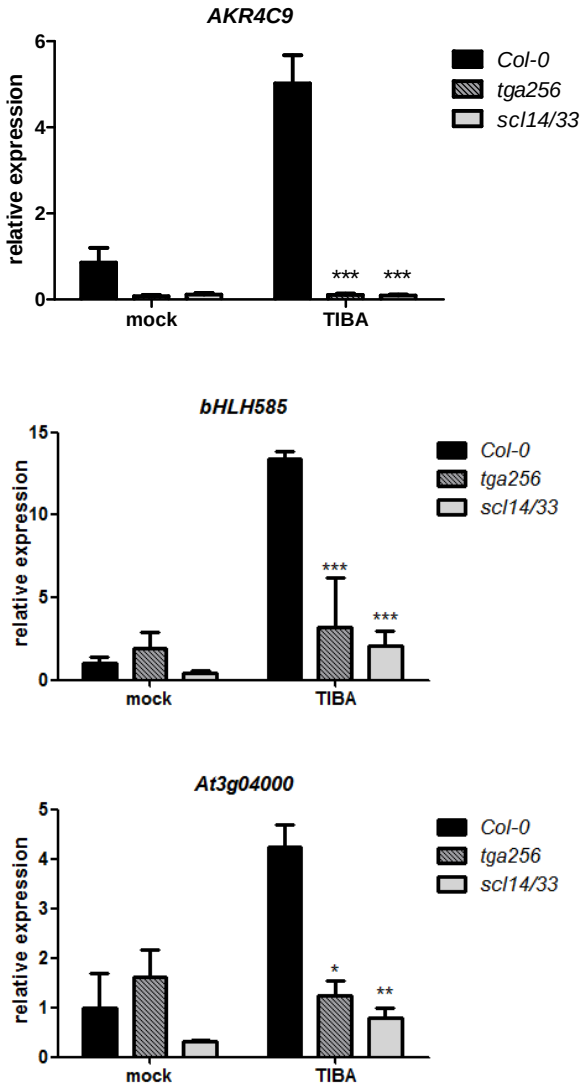
Figure S1: Sequence similarities between the ATAF-type NAC TFs

```
ANAC032   1   ----------------------------------------MMKSGADLQFPPGFRFHPTDEELVLMYLCRKCASQPIPAP  40
ATAF1     1   ----------------------------------------MSEL---LQLPPGFRFHPTDEELVMHYLCRKCASQSIAVP  37
ATAF2     1   ----------------------------------------MKSELNLPAGFRFHPTDEELVKFYLCRKCASEQISAP  37
ANAC102   1   mdfalfssisifeinhkdppirrfiktqnrilstrkqqgtFPKMKAELNLPAGFRFHPTDEELVKFYLCRRCASEPINVP  80

ANAC032   41  IITELDLYRYDPWDLPDMALYGEKEWYFFSPRDRKYPNGSRPNRAAGTGYWKATGADKPIGRPKPVGIKKALVFYSGKPP  120
ATAF1     38  IIAEIDLYKYDPWELPGLALYGEKEWYFFSPRDRKYPNGSRPNRSAGSGYWKATGADKPIGLPKPVGIKKALVFYAGKAP  117
ATAF2     38  VIAEIDLYKFNPWELPEMSLYGEKEWYFFSPRDRKYPNGSRPNRAAGTGYWKATGADKPIGKPKTLGIKKALVFYAGKAP  117
ANAC102   81  VIAEIDLYKFNPWELPEMALYGEKEWYFFSHRDRKYPNGSRPNRAAGTGYWKATGADKPIGKPKTLGIKKALVFYAGKAP  160

ANAC032   121 NGEKTNWIMHEYRLADVDRSVR--KKNSLRLDDWVLCRIYNKKGVIEKRR--------SDIEDGLKPVTDTCPP------  184
ATAF1     118 KGEKTNWIMHEYRLADVDRSVR-KKKNSLRLDDWVLCRIYNKKGATERRGPPppvvygDEIMEEKPKVTEMVMPPppqqT  196
ATAF2     118 KGIKTNWIMHEYRLANVDRSASVNKKNNLRLDDWVLCRIYNKKGTMEKYFPA----------DEKPRTTTMAEQS----S  183
ANAC102   161 KGIKTNWIMHEYRLANVDRSASTNKKNNLRLDDWVLCRIYNKKGTMEKYLPA----------------AAEKP----T  218

ANAC032   185 ---------ES-VARL----IS---GSEQAVSPEFTC-----------------SNGRLSNALDFPFNYVDAIADNEIV  229
ATAF1     197 SEFayfDTSDS-VPKLHTIDSS---CSEQVVSPEFTS----EVQSEPKWkdwsavSNDN-NNTLDFGFNYIDAIVDNAFG  267
ATAF2     184 SPF---DTSDSTYPTLQEDDSSssgGHGHVVSPDVL-----EVQSEPKW--------GELEDALE-AF--DTSMFGSSME  244
ANAC102   219 EKM---STSDSRCSS-------------HVISPDVTCsdnwEVESEPKW--------INLEDALE-AFNDDTSMF-SSIG  272

ANAC032   230 SRLLGG--NQMWSTTLDPLVVRQGTF--------------  253
ATAF1     268 G---GGSSNQMFPLQ-DMFMYMQKPY--------------  289
ATAF2     245 LLQPDAFVPQFLYQS-DYFTSFQDPPEQKPFLNWSFAPQG  283
ANAC102   273 LLQNDAFVPQFQYQSSDFVDSFQDPFEQKPFLNWNFAPQG  312
```



Multiple sequence alignment was performed using COBALT (Papadopoulos and Agarwala, 2007). In the top figure, columns with no gaps are colored in blue or red. The red color indicates highly conserved columns and blue indicates less conserved ones. The bottom figure indicates evolutionary distances between sequences modeled using the Neighbour-joining Tree method. The ATAF-type TFs (belonging to sub-group NAC-a-9) are within the yellow box while others are remaining members of the NAC-a sub-family.

Figure S2: Induction of *AKR4C9*, *bHLH585* and *At3g04000* by TIBA requires TGA256 and SCL14/33 proteins

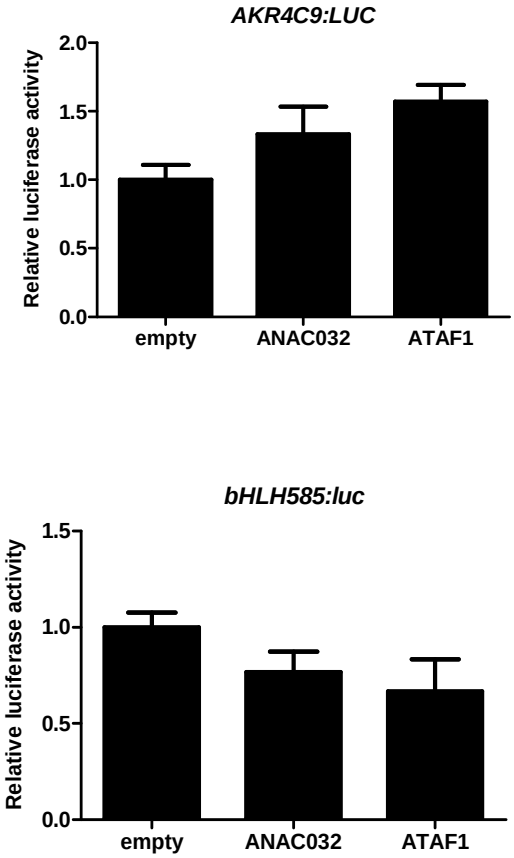


Six-week old soil-grown (under short day conditions) *Col-0*, *tga256* and *scl14/33* plants were treated with 0.1mM TIBA for 8 hours. Treatment with 0.1% DMSO served as control. The relative transcript levels (normalized to house-keeping gene UBQ5) of *AKR4C9, bHLH585* and *At3g04000* were determined by qRT-PCR. The average relative expression in mock treated *Col-0* plants was set to 1. The mean values (±SE) from five individual plants are shown. Asterisks indicate significant differences compared with wild type (Two-way ANOVA; ***P<0.001, **P<0.01, *P<0.05)

Figure S3: Presence of ATAF1-binding consensus sequences as described by Jensen et al., 2013 in the promoters of the three target genes

The promoter sequences (-1000bp region) of the three target genes are shown. The highlighted sequences indicate conserved ATAF1-binding sequences as described by Jensen et al., 2013. Sequences highlighted in green are conserved 6-mers while those in yellow indicate conservation of 5bp out of the 6-mer described. The consensus sequence described for ATAF1 is T[A/C/G]CGT[A/G] and TT[A/C/G]CGT.

Figure S4: ANAC032 and ATAF1 without additional VP16 domain is unable to induce *AKR4C9* and *bHLH585* promoter constructs



Leaves from four-week old soil-grown *ataf1anac032* plants grown under 12-h light/12-h dark photoperiod were used for protoplast isolation and transformation with different effector and reporter constructs as indicated in the graphs. The values shown are means of two independent experiments, each with 4-5 reaction per effector construct. Statistical analysis using one-way ANOVA indicates no significant differences between the reactions.

10. Abbreviations

%	Percent
°C	degree Celsius
µl	Microliter
µM	micromolar
A. thaliana	*Arabidopsis thaliana*
A. tumefaciens	*Agrobacterium tumefaciens*
ABA	Abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
AKR	Aldo-Keto Reductase
AKR4C9	ALDO KETO REDUCTASE FAMILY 4 MEMBER C9
ANAC032	A. thaliana NAC DOMAIN CONTAINING PROTEIN 32
ANOVA	Analysis of Variance
AOS	ALLENE OXIDE SYNTHASE
as-1	activation sequence-1
ATAF	ARABIDOPSIS THALIANA ACTIVATION FACTOR
B. cinerea	*Botrytis cinerea*
bHLH	basic Helix-Loop-Helix
bZIP	basic domain/leucine zipper
$CaCl_2$	calcium chloride
cDNA	complementary DNA
cm	centimeter
COI1	CORONATINE INSENSITIVE 1
COR78	COLD REGULATED 78
C_t	cycle threshold
CUC2	CUP-SHAPED COTYLEDON 2
DBD	DNA-binding domain
DDE2	DELAYED DEHISCENCE 2
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
dpi	day(s) post infection
E.coli	*Escherichia coli*
e.g.	for example (exempli gratia)
EDTA	Ethylene di-amine tetra-acetic acid
EIN3	ETHYLENE INSENSITIVE 3
ET	ethylene
EtBr	ethidium bromide
EtOH	ethanol
FLC	FLOWERING LOCUS C
fwd	forward
GA	gibberellic acid
GO	Gene Ontology
GRAS	GAI, RGA, SCR
GUS	β-Glucuronidase
h	hour
HCl	hydrochloric acid
HPLC-MS/MS	High Performance Liquid Chromatography-Tandem Mass Spectrometry

HSPB	High Salt Precipitation Buffer
JA	jasmonic acid
JA-Ile	(+)-7-iso-Jasmonyl-L-isoleucine
JAZ10	JASMONATE ZIM DOMAIN PROTEIN 10
KCl	potassium chloride
l	litre
LB	Luria Bertani
LD	long day
M	Molarity
MeJA	methyl jasmonate
MES	2-[N-Morpholino]-ethanesulfonic acid
mg	milligram
$MgCl_2$	magnesium chloride
min	minute
ml	milliliter
mM	millimolar
mRNA	messenger RNA
MS	Murashige and Skoog medium
MUG	4-methyl-umbelliferyl-ß-D-glucuronide
Na_2CO_3	sodium carbonate
NAC	NAM, ATAF1/2, CUC2
NaCl	sodium chloride
NAM	NO APICAL MERISTEM
ONPG	o-nitrophenyl-β-D-galactopyranoside
OPDA	12-oxo-phytodienoic acid
P	P-value (probability of obtaining a test statistic assuming that the null hypothesis is true)
P. xylostella	*Plutella xylostella*
PCR	polymerase chain reaction
PDB	potato dextrose broth
PDF1.2	PLANT DEFENSIN 1.2
pH	negative logarithm of the activity of the (solvated) hydronium ion
PR-1	PATHOGENESIS RELATED-1
qRT-PCR	quantitative Real-time PCR
rev	reverse
RNA	ribonucleic acid
RNase	ribonuclease
rpm	rotations per minute
RT	reverse transcriptase
RT	room temperature
s	second
S. cervisiae	*Saccharomyces cervisiae*
SA	salicylic acid
SAG	salicylic acid-2-O-β-D-glucoside
SCL14	SCARECROW LIKE-14
SD	short day
SDS	sodium dodecylsulfate
SE	standard error of mean
TAE	Tris-acetate-EDTA
taq	*Thermus aquaticus*
TF	transcription factor
TGA	TGACG motif binding protein

TIBA	2,3,4-triiodobenzoic acid
TR domain	transcriptional regulatory domain
UBQ5	UBIQUITIN-5
VSP2	VEGETATIVE STORAGE PROTEIN 2
w/v	weight per volume
WT	wild-type
X-Gluc	bromo-4-chloro-3-indolyl-β-D-glucuronide
YPAD	Yeast extract-peptone-adenine-dextrose

11. Curriculum Vitae

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Academic Qualifications and Research Experience

April 2010 – April 2014	PhD degree in Biology at Georg-August-Universität Göttingen, Germany Research on " Identification of the role of *Arabidopsis* ATAF-type NAC transcription factors in plant stress and development "
Jan 2009 – March 2010	Junior Research Fellow (CSIR-JRF) at Biochemistry Department, National Chemical Laboratory, Pune, India Research on " Application of RNAi technology as a means to control the pest *Helicoverpa armigera* "
2006-2008	Master of Science (M.Sc) in Microbiology from University of Pune, India (obtained 5.56 GPA on a scale of 6.0) M.Sc Project: " Characterization of Extended-Spectrum-β-Lactamase and Metallo-β-Lactamase producers from clinical isolates of *Acinetobacte*r sp. "
2003-2006	Bachelor of Science (B.Sc) in Microbiology from Abasaheb Garware College, Pune, India (obtained 78.65%)
2003	H.S.C (Higher Secondary Certificate) from Abasaheb Garware College, Pune, India (obtained 84.50%)
2001	S.S.C (Secondary School Certificate) from Vidya Bhavan High School, Pune, India (obtained 83.86%)