Regulation of Agrobacterial Oncogene Expression in Host Plants

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Contents

Abstract

Virulent *Agrobacterium tumefaciens* strains transfer and integrate a DNA region of the tumor-inducing (Ti) plasmid, the T-DNA, into the plant genome and thereby cause crown gall disease. The most essential genes required for crown gall development are the T-DNA-encoded oncogenes, *IaaH* (indole-3-acetamide hydrolase), *IaaM* (tryptophan monooxygenase) for auxin, and *Ipt* (isopentenyl transferase) for cytokinin biosynthesis. When these oncogenes are expressed in the host cell, the levels of auxin and cytokinin increase and cause cell proliferation. The aim of this study was to unravel the molecular mechanisms, which regulate expression of the agrobacterial oncogenes in plant cells. Transcripts of the three oncogenes were expressed in *Arabidopsis thaliana* crown galls induced by *A. tumefaciens* strain C58 and the intergenic regions (IGRs) between their coding sequences (CDS) were proven to have promoter activity in plant cells. These promoters possess eukaryotic sequence structures and contain *cis*-regulatory elements for the binding of plant transcription factors. The high-throughput protoplast transactivation (PTA) system was used and identified the *Arabidopsis thaliana* transcription factors WRKY18, WRKY40, WRKY60 and ARF5 to activate the *Ipt* oncogene promoter. No transcription factor promoted the activity of the *IaaH* and *IaaM* promoters, despite the fact that the sequences contained binding elements for type B ARR transcription factors. Likewise, the treatment of *Arabidopsis* mesophyll protoplasts with cytokinin (*trans*-zeatin) and auxin (1-NAA) exerted no positive effect on *IaaH* and *IaaM* promoter activity. In contrast, the *Ipt* promoter strongly responded to a treatment with auxin and only modestly to cytokinin. The three *Arabidopsis* WRKYs play a role in crown gall development as the *wrky* mutants developed smaller crown galls than wild-type plants. The *WRKY40* and *WRKY60* genes responded very quickly to pathogen infection, two and four hours post infection, respectively. Transcription of the *WRKY18* gene was induced upon buffer infiltration, which implicates a response to wounding. The three WRKY proteins interacted with ARF5 and with each other in the plant nucleus, but only WRKY40 together with ARF5 increased activation of the *Ipt* promoter. Moreover, ARF5 activated the *Ipt* promoter in an auxin-dependent manner. The severe developmental phenotype of the *arf5* mutant prevented studies on crown gall development, nevertheless, the reduced crown gall growth on the transport inhibitor response 1 (TIR1) *tir1* mutant, lacking the auxin sensor, suggested that auxin signaling is required for optimal crown gall development. In conclusion, *A. tumefaciens* recruits the pathogen defense related WRKY40 pathway to activate *Ipt* expression in T-DNA-transformed plant cells. *IaaH* and *IaaM* gene expression seems not to be controlled by transcriptional activators, but the increasing auxin levels are signaled via ARF5. The auxin-depended activation of ARF5 boosts expression of the *Ipt* gene in combination with WRKY40 to increase cytokinin levels and induce crown gall development.

Zusammenfassung

Virulente Bakterien des Stamms *Agrobakterium tumefaciens*, transferieren und integrieren einen Teil ihrer DNA, die T-DNA aus dem Tumor induzierenden Plasmid (Ti), in das Pflanzengenom. Dadurch wird die Tumorbildung induziert und die Krankheit bricht aus. Die wichtigsten Gene, die für die Entwicklung eines Tumors benötigt werden, sind auf der T-DNA lokalisierte Onkogene: IaaH (indole-3-aceetamide hydrolase), IaaM (tryptophan monooxygenase) für die Auxin Biosynthese und Ipt (isopentenyl transferase) für die Cytokinin Biosynthese. Werden diese Onkogene in der Wirtszelle exprimiert, steigt der Gehalt an Auxin und Cytokinin und fördert die Zellteilung. Das Ziel dieser Arbeit war es die molekularen Mechanismen, die die Expression der agrobakteriellen Onkogene in Pflanzenzellen regulieren, aufzuklären. Transkripte der drei Onkogene wurden in Tumoren an *Arabidopsis thaliana* exprimiert. Die Tumore wurden durch den *A. tumefaciens* Stamm C58 induziert. Dabei konnte gezeigt werden, dass die Sequenzabschnitte zwischen den Onkogenen (IGRs: intergenic regions) eine Promoteraktivität in der Pflanzenzelle besitzen. Diese Promoter haben eukaryotische Sequenzstrukturen und enthalten *cis*-Elemente, an die pflanzliche Transkriptionsfaktoren binden. Mit Hilfe der PTA (high-throughput protoplast transactivation) Methode wurden die pflanzlichen Transkriptionsfaktoren WRKY18, WRKY40, WRKY60 und ARF5 von *Arabidopsis thaliana* identifiziert, welche den Promoter des *Ipt* Onkogens aktivieren. Für *IaaH* und *IaaM* konnte kein Transkriptionsfaktor, der die Promotersequenzen aktiviert, identifiziert werden, obwohl die Promotersequenzen Bindedomänen für den Typ B ARR Transkriptionsfaktor enthalten. Ebenso zeigte die Behandlung von *Arabidopsis* Protoplasten aus dem Mesophyll mit Cytokinin (*trans*-zeatin) und Auxin (1-NAA) keinen positiven Effekt auf die Aktivität des *IaaH* und des *IaaM* Promoters, wohingegen der *Ipt* Promoter stark auf eine Behandlung mit Auxin und leicht auf eine Behandlung mit Cytokinin reagierte. Die drei WRKYs aus *Arabidopsis* spielen eine Rolle in der Tumorentwicklung, da die *wrky* Mutante kleinere Tumore zeigt, als die Wild Typ Pflanzen. Die Gene *WRKY40* und *WRKY60* reagieren sehr schnell, innerhalb von zwei, beziehungsweise vier Stunden, auf eine Pathogen Infektion. Die Transkription des *WRKY18* Gens wurde durch die Infiltration von Puffer in Blätter induziert, dies lässt auf eine Reaktion im Zusammenhang mit Wunderzeugung schließen. Die drei WRKY Proteine interagieren mit einander und mit ARF5 im Zellekern der Pflanzenzelle, aber nur WRKY40 und ARF5 können gemeinsam den *Ipt* Promoter aktivieren. Zusätzlich kann ARF5 den *Ipt* Promoter, in Abhängigkeit von Auxin, aktivieren. Wegen starker Entwicklungsstörungen der *arf5* Mutante, konnte das Tumorwachstum an dieser Mutante nicht untersucht werden. Das reduzierte Tumorwachstum an der *tri1* (transport inhibitor response, TIR) Mutante, der ein Auxinsensor fehlt, deutet auf die Notwendigkeit des Auxinsignalwegs für optimales Tumorwachstum hin. Zusammengefasst benutzt *A. tumefaciens* den *WRKY40* Signalweg, der mit der Pathogen Abwehr verbunden ist, um die *Ipt* Expression in der mit T-DNA transformierten Pflanzenzelle zu aktivieren. Die Genexpression von *IaaH* und *IaaM* schein nicht von Transkriptionsfaktoren abhängig zu sein, aber erhöhte Auxin Werte

werden von ARF5 erkannt. Die Auxin abhängige Aktivierung von ARF5 verstärkt die Expression des *Ipt* Gens gemeinsam mit WRKY40 um die Cytokin Werte in der Pflanzenzelle zu erhöhen und somit die Tumorentwicklung einzuleiten.

1. Introduction

Agrobacterium tumefaciens is used as tool for plant transformation since 1980s, but in nature it is a pathogenic bacterium that infects several plant species and causes the crown gall disease. The crown gall disease is widespread among grapes, fruit and nut trees, cranberries, chrysanthemum, roses, and other nursery crops. As early as 1853, the crown gall was firstly reported as neoplastic disease of plants [1]. *A. tumefaciens* was isolated from Paris daisy (*Argyranthemum frutescens*) and identified as the causal agent of the crown gall disease in 1907 [2]. The crown gall tumor cells were considered to be transformed from normal plant cells by a bacterium-derived "tumor-inducing principle" (TIP) in 1947 [3]. In 1974 it was proposed that a large plasmid, the Ti (tumor inducing) plasmid from *A. tumefaciens,* could be the TIP [4]. It was demonstrated that TIP was a region of the *A. tumefaciens* Ti plasmid in 1977 [5]. This region was called T-DNA (transferred-DNA) and it could be transferred and integrated into the plant genome in the crown gall tumor cells. Later, the Ti plasmid was constructed as tool for transformation of any DNA of interest into plants and finally, was used to generate the first transformed plant [6].

1.1 Ti plasmid

The Ti plasmid represents extrachromosomal DNA in *A. tumefaciens* cells and is required for tumorigenesis. The size of the Ti plasmid is often more than 200 kb large, and, pTiC58 from *A. tumefaciens* strains C58, the first Ti plasmid to be sequenced, consists of 214,233 bp [7,8]. There are two important regions in a Ti plasmid, the "virulence" (*vir*) and T-DNA region (Figure 1A). The genes located in the *vir* region encode Vir proteins, which function in the process of T-DNA transfer and integration. VirD2 associates with the T-strand which is excised by VirD1/VirD2 from the T-DNA region. The T-strand conjugated with VirD2 is transferred with VirE2, VirE3 and VirF into the plant cell by the type IV secretion system, and then coated by VirE2 to form the mature T-complex. The T-complex is transported into the plant cell nucleus through the nuclear pore complex. VirE3 and VirF are involved in integration of the T-DNA into the plant genome [9,10]. The genes of the T-DNA region can be classified into two groups [11]. One group of T-DNA genes is responsible for producing opines which provide carbon and nitrogen sources for *A. tumefaciens* [11]. The other group of T-DNA genes is responsible for crown gall tumor development and called oncogenes (1.2). Opine-related genes encode proteins for the synthesis and secretion of amino acid-sugar conjugates, termed as opines. *A. tumefaciens* strains and their Ti plasmids can be classified by the opines they metabolize. The most common opines are octopine, nopaline, agropine and succinamopine. The Ti plasmid used in this study is, pTiC58, a nopaline Ti plasmid [7,8].

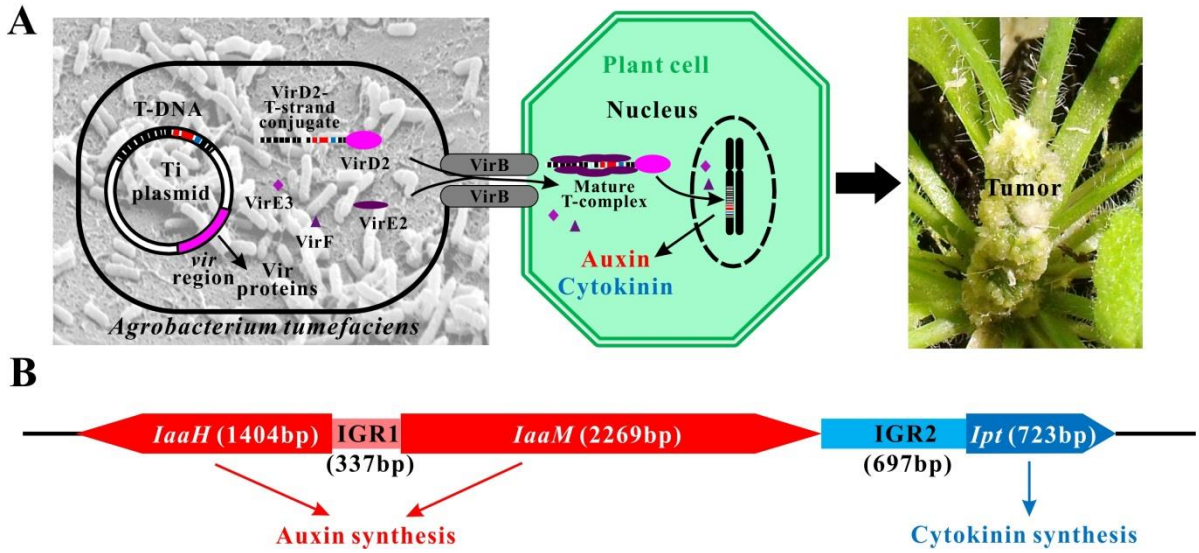


Figure 1. Agrobacterial oncogenes induce crown gall tumor development.
(A) Overview over the plant transformation process by *Agrobacterium tumefaciens* and a crown gall tumor *on Arabidopsis thaliana* stems. The T-strand is excised by VirD1/VirD2 from the T-DNA region of Ti plasmid and conjugated with VirD2. The VirD2 T-strand conjugate and Vir proteins are transported into plant cells via the type_IV secrtion system which mainly consists of VirB. VirE2 covers the T-strand to form the mature T-complex. The T-complex is shuttled into the nucleus with VirE3 and VirF through the nuclear pore complex, and then integrated into plant genome. Expression of the oncogene encoded proteins involved in auxin and cytokinin synthesis causes accumulations of auxin and cytokinin in T-DNA transformed plant cells and contribute to crown gall development. (B) Scheme of *IaaH*, *IaaM* and *Ipt* oncogene coding sequence (CDS) and the two intergenic regions (IGRs) between them in the T-DNA region of pTiC58, a nopaline Ti plasmid in *A. tumefaciens* strains C58.

1.2 The agrobacterial oncogenes

The agrobacterial oncogenes are mainly involved in the synthesis of phytohormones, including the enzymes IaaM, IaaH, Ipt, gene 6b and gene 5.

IaaH and *IaaM* were named as *tms* (tumor morphology shooty, *IaaH/tms2* and *IaaM/tms1*) in 1980s, because the *tms* mutants generated crown gall tumors which differentiated into shoots [12]. The *tms* mutants produced crown gall tumors with reduced auxin levels [13], which suggested that *IaaH* and *IaaM* were involved in auxin synthesis signaling. *IaaM* encodes a tryptophan monooxygenase that converts tryptophan (Trp) into indole-3-acetamide (IAM), and *IaaH* encodes an indole-3-acetamide hydrolase, which converts IAM into indole-3-acetic acid (IAA) [14-16]. Plants have no homologous genes to *IaaH* and *IaaM*, but some auxin-producing plant pathogens, such as *Pseudomonas savastanoi* pv. *savastanoi* and *Erwinia herbicola* pv. *Gypsophilae*, have genes with homology to *IaaH* and *IaaM* [17,18]. This phenomenon suggests that these two oncogenes might be of prokaryotic origin, but are expressed in eukaryotic plant cells. The IAM-dependent IAA biosynthesis pathway is different from the plant's endogenous Trp-dependent pathway. Therefore, *IaaH* and *IaaM*, which are transferred via agrobacterial T-DNA into the plant genome, mediate an unusual and probably uncontrolled auxin biosynthesis pathway.

The former name of *Ipt* oncogene is *tmr* (tumor morphology rooty). The *tmr* mutants produced small and rooty tumors with low levels of the cytokinin type zeatin [12,13]. *Ipt* produces an isopentenyl transferase (Ipt), which catalyzes the rate-limiting step in cytokinin biosynthesis [14-16]. The *Ipt* encoded protein condenses adenosine monophosphate (AMP) and isopentenyl pyrophosphate (iPePP) to isopentenyl adenosine 5'-monophosphate (iPMP), or condenses AMP and 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate (HMBDP) to zeatin-riboside 5'-monophosphate (ZMP), and finally host enzymes convert iPMP or HMBDP into the cytokinin zeatin [11,19,20]. In *Arabidopsis thaliana*, there are nine isopentenyl transferase-encoding genes, *AtIPT1 to AtIPT9* [21,22]. However, AtIPT proteins convert the substrate dimethylallyl diphosphate (DMAPP) to isopentenyl adenosine 5'-monophosphate (iPMP) [23]. Therefore, agrobacterial *Ipt* oncogene and host *AtIPT* genes mediate totally different cytokinin biosynthesis pathways.

The phenotypes of *tml* (tumor morphology large) mutants are different depending on opine types. The octopine *A. tumefaciens* strains *tml* mutants generated larger crown gall tumors [24], but the nopaline *A. tumefaciens* strains *tml* mutants had no function on tumor size [25]. The *tml* locus contains two genes: *gene 6a* and *gene 6b*. The *gene 6a* did not play any roles in crown gall development [26], so it is not defined as an oncogene. The function of *gene 6b* has not been determined so far. *Gene 6b* likely affects the sensitivity of plant cells to phytohormones [11,19,20]. Another oncogene, *gene 5*, encodes an enzyme, which synthesizes indole-3-lactate (ILA), an antagonistic auxin analogue [11,19,20]. It is believed that *gene 6b* and *gene 5* play non-essential roles in crown gall development [20].

As mentioned above, the most important oncogenes are *IaaH, IaaM and Ipt*. The multistep process of *Agrobacterium*-mediated genetic transformation has been studied for decades (Figure 1) [9,10]. However, the molecular mechanism of regulation of oncogene expression in plant cells is still scant.

1.3 Auxin and cytokinin

The plant tissues, which are transformed by *A. tumefaciens* with a virulent T-DNA, accumulate phytohormones such as auxin, cytokinin, abscisic acid (ABA), salicylic acid (SA) and ethylene (ET) [27,28]. Auxin and cytokinin act as the crucial modulator of the plant-*Agrobacterium* relationship. Pretreatment of auxin and cytokinin promotes transformation efficiency and tumorigenesis of *A. tumefaciens* [29-31].

1.3.1 Auxin

The word "auxin" is derived from the Greek word "auxein" which means to grow or increase. Auxin is a kind of plant growth hormone, which plays roles in many process of plant development, for example cell enlargement, cell division, vascular tissue differentiation, root initiation, tropistic responses, apical dominance and organ growth [32].

1.3.1.1 Chemical forms of auxins

The natural chemical forms of auxins in plants are the free acid and conjugated forms. Free acids are indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), 4-chloroindole-3-acetic acid (4-Cl-IAA) and phenylacetic acid (PAA) [32]. Free IAA is the main natural form in most plants. IBA is a kind of commercial auxin for plant propagation, but it is also naturally present in plants [33]. In addition, 4-Cl-IAA is also reported to be a natural auxin [32]. Besides indolic auxin, phenylacetic acid (PAA) has low auxin activity in some plants too [34]. The main fraction of the auxin in plants is conjugated to sugars, sugar alcohols, amino acids or proteins, as for example IAA-aspartate (IAA-Asp). Auxin is inactivated by conjugation, and thereby the plants can keep a homeostatic level of active and inactive auxin. In addition to the natural forms of auxins, many synthetic forms have been used commercially in plant tissue cultures for decades, for example 1-Naphthaleneacetic acid (1-NAA) and 2,4-Dichlorophenoxyacetic acid (2, 4-D).

1.3.1.2 Auxin biosynthesis

Tryptophan (Trp) is the main precursor for IAA biosynthesis in plants. Four pathways for IAA biosynthesis from Trp in plants are proposed (Figure 2A): (*i*) the indole-3-acetaldoxime (IAOx) and glucosinolate pathway, (*ii*) the YUCCA (YUC) pathway, (*iii*) the indole-3-pyruvic acid (IPA) pathway and (*v*) the indole-3-acetamide (IAM) pathway [35]. The IAOx and glucosinolate pathway was only found in few plant species which include the CYP79 genes, as for example in *Arabidopsis* [36]. The IAM pathway is only known as IaaM/IaaH pathway in plant crown gall tissues transformed by *A. tumefaciens* [35]. The YUC and IPA pathway are the most important in plants. Previously it has been proposed that the YUC pathway involves the YUC genes encoding flavin monooxygenase-like proteins that catalyze the conversion of tryptamine (TAM) to N-hydroxy-TAM (HTAM) for IAA synthesis. In the IPA pathway, IPA is synthesized from Trp by TAA1 (tryptophan aminotransferase of *Arabidopsis*) [35]. However, more recently it was demonstrated that YUC and IPA are involved in the same IAA biosynthesis pathway (Figure 2B) [37]. In this IPA-YUC pathway, IPA is synthesized from Trp by TAA1, and then IPA is converted into IAA by YUC [37].

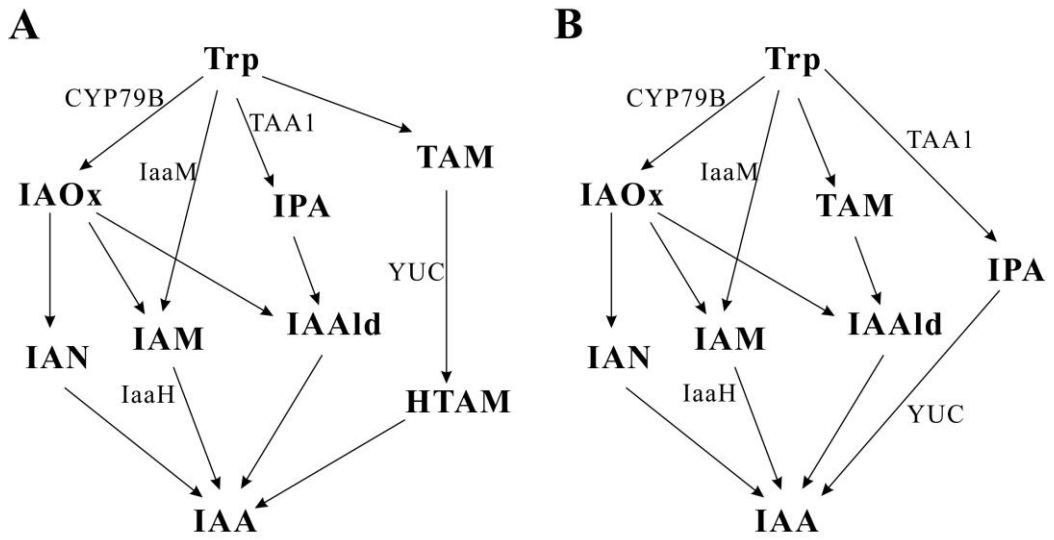


Figure 2. Two proposed IAA biosynthesis pathways in plants.
(A) Previously proposed IAA biosynthesis pathway and (B) very recently proposed IAA biosynthesis pathway [37]. Modified from the model of Kiyoshi Mashiguchia et al [37]. The details see 1.3.1.2.

In addition to plants, IAA is also found in some plant-associated bacteria. It is estimated that 80% of all soilborne bacteria can produce IAA [32]. Previously it has been shown that both *A. tumefaciens* strains, C58 and GV3101, produce IAA and secrete it into the culture medium [28]. However, the role of IAA produced by bacteria and the mechanism of IAA biosynthesis in bacteria is not completely clear.

1.3.1.3 Auxin signaling in plant pathogen defense

Auxin plays a role in plant growth and development however it is also involved in plant-pathogen interaction. Previous studies suggested that auxin signaling promotes resistance against necrotrophs but susceptibility to biotrophs [38]. It is likely that auxin regulates plant-pathogen interaction via different mechanisms. Auxin promotes susceptibility to biotrophic pathogen through repression of SA signaling [39]. It is reviewed that auxin pathways also interacts other defense signaling pathways, for example jasmonic acid (JA) and ethylene (ET) [38,40]. The physiological effects of auxin on plants may contribute to plant defense. For example, auxin impairs distribution and opening of stomata which are the entry points for some pathogen [41]; auxin may be involved in programmed cell death and the hypersensitive response (HR) [42]. Auxin may also influence biosynthesis of defense-related compounds. The auxin response factors ARF1 and ARF9 negatively regulate glucosinolate accumulation, and ARF9 positively influences camalexin accumulation [43].

1.3.1.4 Auxin in crown gall tumors

The concentration of free auxin (free IAA) in plant tissue is usually not high, but it increases after inoculation of *A. tumefaciens*. The level of free IAA in the crown gall tumors is up to 13-fold higher than in the control tissue two weeks after inoculation of *Ricinus* hypocotyls [27]. In *Arabidopsis*, the

levels of free IAA in the infected plants increases more than two-fold at six days after inoculation compared to mock-treated tissues. The developing tumors accumulate four-fold more of free IAA compared to the control tissue [28].

1.3.2 Cytokinin

Cytokinin is also a plant growth hormone that has effects on cell division, morphogenesis, growth of lateral buds, leaf expansion, leaf senescence, chloroplast development and many other development processes.

1.3.2.1 Chemical forms of cytokinin

There are two types of cytokinins: adenine-type cytokinins and phenylurea-type cytokinins [44]. The phenylurea-type cytokinins such as *N,N'*-phenylurea (DPU), *N*-phenyl-*N'*-(2-chloro-4-pyridyl)urea (CPPU) and *N*-phenyl-*N'*-(1,2,3-thidiazol-4-yl)urea (thidiazuron,TDZ) are synthetic and unnatural cytokinins [44]. The natural adenine-type cytokinins include *trans*-zeatin (tZ), *cis*-zeatin (cZ), dihydrozeatin (DZ), and isopentenyl adenine (iP) [23,45]. The latter, free base cytokinins, are considered as active forms, and the most common form in plants is *trans*-zeatin (tZ) [44]. The nucleoside forms of cytokinin represented by isopentenyl adenine riboside (iPR) and *trans*-zeation riboside (tZR) function as translocation forms in plants, whereas the sugar conjugation forms are the storage and inactivated forms [44]. Except for natural cytokinins, kinetin and 6-benzylaminopurine (6-BA) are usually used as synthetic cytokinins in cell or tissue cultures.

1.3.2.2 Cytokinin biosynthesis

There are two proposed pathways for cytokinin biosynthesis: the tRNA pathway and free adenine nucleotide pathway. The initial and rate-limiting steps in both cytokinin biosynthesis pathways are catalyzed by isopentenyl transferase (IPT) [23,44]. In the free adenine nucleotides pathway, plant IPT catalyzes dimethylallyl diphosphate (DMAPP) and APM, ADP or ATP condensation to form isopentenyl adenine riboside 5'-monophosphate (iPRMP), isopentenyl adenine riboside 5'-diphosphate (iPRDP) or isopentenyl adenine riboside 5'-triphosphate (iPRTP), respectively [23,44]. The three products are also named isopentenyl adenosine 5'-monophosphate (iPMP), isopentenyl adenosine 5'-diphosphate (iPDP) or isopentenyl adenosine 5'-triphosphate (iPTP) in some publications. Furtheron, the cytochrome P450 mono-oxygenases CYP735A1 and CYP735A2 convert iPRMP, iPRDP or iPRTP into tZRMP, tZRDP or tZRTP respectively [46]. The active forms of cytokinin, iP and tZ, are generated from iPRMP and tZRMP, respectively by the cytokinin nucleoside 5-monophosphate phosphoribohydrolase called LOG [47]. In the tRNA cytokinin biosynthesis pathway, tRNA and DMAPP are coupled, affording isopentenylated tRNA, by a condensation reaction catalyzed by tRNA-IPT [23,44].

The *Arabidopsis* genome contains nine *IPT* genes, *AtIPT1 to AtIPT9* [21,22]. *AtIPT2* and *AtIPT9* encode tRNA-IPT enzymes, and the others (*AtIPT1*, *AtIPT3 to AtIPT8*) encode IPT enzymes [44]. The

agrobaterial oncogene encoding Ipt is similar to the plant IPT, but the agrobacterial Ipt enzyme uses isopentenyl pyrophosphate (iPePP) or 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate (HMBDP) instead of DMAPP as substrates to synthesize zeatin-riboside 5'-monophosphate (ZMP) [11,19,20].

1.3.2.3 Cytokinin signaling in plant pathogen defense

Cytokinin, like auxin, plays a role in plant-pathogen-interaction as well as plant development [48]. It is considered that the cytokinins produced by many biotrophic or hemibiotrophic pathogens promote their proliferation in host plants [48]. Cytokinin promotes transformation efficiency and tumorigenesis of *A. tumefaciens* [29-31]. Cytokinins produced by the hemibiotrophic actinomycete *Rhodococcus fascians* are recognized by the *Arabidopsis* cytokinin receptors AHK3 and AHK4 and are essential for symptom development [49]. On the other hand, it was reported that plant endogenous cytokinins enhance resistance of plants to pathogen infections. For example, transgenic tobacco plants with high levels of endogenous cytokinins induce a hypersensitive-like response and increases activity of stress responsive enzymes [50,51]. Furthermore, it was found that plant-derived cytokinins enhance resistance of *Arabidopsis* to *Pseudomonas syringae* pv. *tomato* DC3000 via SA signaling [52]. However, later on it was reported that cytokinins promote resistance of tobacco to *Pseudomonas syringae* pv *tabaci* through elevated phytoalexin synthesis independent of SA signaling [53].

1.3.2.4 Cytokinin in crown gall tumors

The level of cytokinin is increased in crown gall tumor tissues of *Ricinus* hypocotyls induced by *A. tumefaciens* compared to the control tissue [27]. The dominating form of cytokinin in this crown gall tZ and tZR, and the highest concentration of tZ and tZR was detected 4 weeks after inoculation [27]. The strong GUS activity of tumor calli containing the cytokinin responsive promoter ARR5 fused to β-glucuronidase (GUS; *ARR5::GUS*) suggests high amounts of active cytokinin in the crown gall tumor [54].

1.4 Features for gene expression in eukaryotic cells

In eukaryotic cells, mRNAs from protein-coding genes are transcribed by RNA polymerase II complex. In addition to RNA polymerase II, the general transcription factors are required for transcriptional initiation. The complex of the general transcription factors and RNA polymerase II recognizes sequence elements in the promoters as for example the transcription start site (TSS) and TATA box [55]. The TATA box is usually localized at −25 bp to −30 bp upstream of the TSS (+1) and is identified by the general transcription factor TFIID (TF, transcription factor; IID, RNA polymerase IID). Besides the TATA box, there is a consensus sequence around the eukaryotic TSS which functions as initiator (Inr) box (YYANWYY, TSS is underlined, Y = C/T, W = A/T, N = A/G/C/T) [56,57]. In addition, −1/+1 positions of plant genes are applied to the plant specific "YR Rule" (YR, TSS is underlined, Y = C/T, R = A/G) [56,57]. The promoter region for binding of the complex of the general transcription factors and RNA polymerase II is the core promoter, which functions in basal

and low level of transcription [55]. In addition to the core promoter, high levels of transcripts require an upstream promoter sequence, which serve as enhancer elements for the binding of transcription factors. For example the CAAT box, which in many eukaryotic promoters is usually localized at −70 bp to −80 bp of the TSS (+1), enhances the promoter activity. Enhancer elements are found in many eukaryotic promoters, and they can localize on the sense or anti-sense DNA strand far away from the TSS [55].

1.5 Plant transcription factors

The expression of genes in different cells at different times in development is a complex process, and this process is mainly regulated by transcription factors. Nearly 10% of the genes encode transcription factors in higher plants. According to the Plant Transcription Factor Database v3.0 (PlantTFDB 3.0) (http://planttfdb.cbi.pku.edu.cn/index.php?sp=Ath), in *Arabidopsis thaliana*, 2296 transcription factors (1716 loci) have been identified and classified into 58 families [58]. The large number of transcription factors contributes to the regulation of almost all processes of the plant life cycle. The function of transcription factors is the result of the binding activity to specific *cis*-regulatory elements in the promoters of plant genes. In general, a typical transcription factor usually contains four domains: (*i*) DNA binding domain (DBD), (*ii*) transcription regulation domain, (*iii*) nuclear localization signals (NLS) and (*iv*) oligomerization domains.

The most important domain is the DNA binding domain, which can be classified into several types of structural motifs, including homeodomain (HD), zinc-finger, winged-helix (WH), basic leucine-zipper (bZIP) and helix-loop-helix (HLH) and so on [59]. Besides these common types of the DNA binding domain, there are some specific domains that are only found in plants, for example AP2/ERF, NAC, WRKY, B3 and SBP [60]. Family classifications of transcription factors depend on the DNA binding domain structure.

Another essential domain of transcription factor is the transcription regulation domain that functions as either repressor or activator. The transcription regulation domain is not as conserved as the DNA binding domain, however, many activation domains contain a specific amino acid rich region. Some types of activation domains of mammalian and yeast, for example SP1 have a glutamine rich domain, CTF/NF-I contain a proline rich domain and the domains of GAL4, VP16 and GCN4 are rich in acidic amino acids (aspartic and glutamic acids). The acidic amino acid rich domains of GAL4 and VP16 are also found in plant transcription factors. Nevertheless, there are still many activation domains without any specific amino acid rich region [59]. The transcriptional activators might interact with the general transcription factors, such as TFIIB or TFIID to promote the transcription of target genes [55]. Some transcription factors contain repression domains and function as repressors via competitive binding with activators for the same *cis*-regulatory elements. Many repression domains are rich in hydrophobic or basic amino acid [59].

The nuclear localization signal (NLS) region is rich in arginine and lysine and plays a role in the process of translocation into the nucleus [61]. One transcription factor can have one or more nuclear localization signals. Some plant transcription factors lack a nuclear localization signal and might enter the nucleus by interacting with other protein [62]. Many transcription factors form hetero- and/or homo-oligomers with members of the same or different families via their oligomerization domains, which influence DNA binding or regulational activity. Thus, oligomerizations of transcription factors increase the gene-control options [59].

1.5.1 The WRKY transcription factor family

1.5.1.1 Protein structure and classification

The first WRKY transcription factor was found in sweet potato in 1994 [63]. The name of WRKY (pronounced "worky") comes from the highly conserved sequence "WRKYGQK", the WRKY domain. The WRKY domain is a part of the DNA binding domain of the WRKY transcription factors, which is present in a conserved polypeptide sequence of about 60 amino acids (Figure 3). Although the "WRKYGQK" sequence is highly conserved, several variant patterns are also found in plants, for example "WRKYGEK" and "WRKYGKK" in rice [64]. Besides the WRKY domain, a zinc finger motif is also found in the DNA binding domain (Figure 3). The DNA binding activity will be abolished if WRKY transcription factors are treated with the divalent metal chelators 1,10-o-phenanthroline or EDTA and prove that a zinc-finger motif is necessary for DNA binding [65].

Figure 3. The WRKY amino acid consensus domains of each WRKY subgroup [66].
The conserved WRKY motif is shown in yellow, and the cysteines and histidines from zinc finger motif are in blue. NT: N-terminal WRKY domain in Group I WRKY members, CT: C-terminal WRKY domain in Group I WRKY members. This figure is modified from the review of Rushton et. al [66].

The members of the WRKY transcription factor family can be classified into three groups according to the number of WRKY domains and the type of the zinc finger motives, and are further divided into nine subgroups (Ia, Ib, IIa, IIb, IIc, IId, IIe, IIIa and IIIb) depending on the primary amino acid sequence (Figure 3, 4). The members of group I have two WRKY domains, of which only the C-terminal WRKY domain has DNA binding activity and the role of the N-terminal WRKY domain is unknown. The members of group II have one WRKY domain and the zinc finger motif C_2H_2(C–$X_{4–5}$–C–$X_{22–23}$–H–X_1–H). Group III WRKY transcription factors have also one WRKY domain, and the C_2HC(C–X_7–C–X_{23}–H–X_1–C) type of zinc finger motif (Figure 4)[66,67].

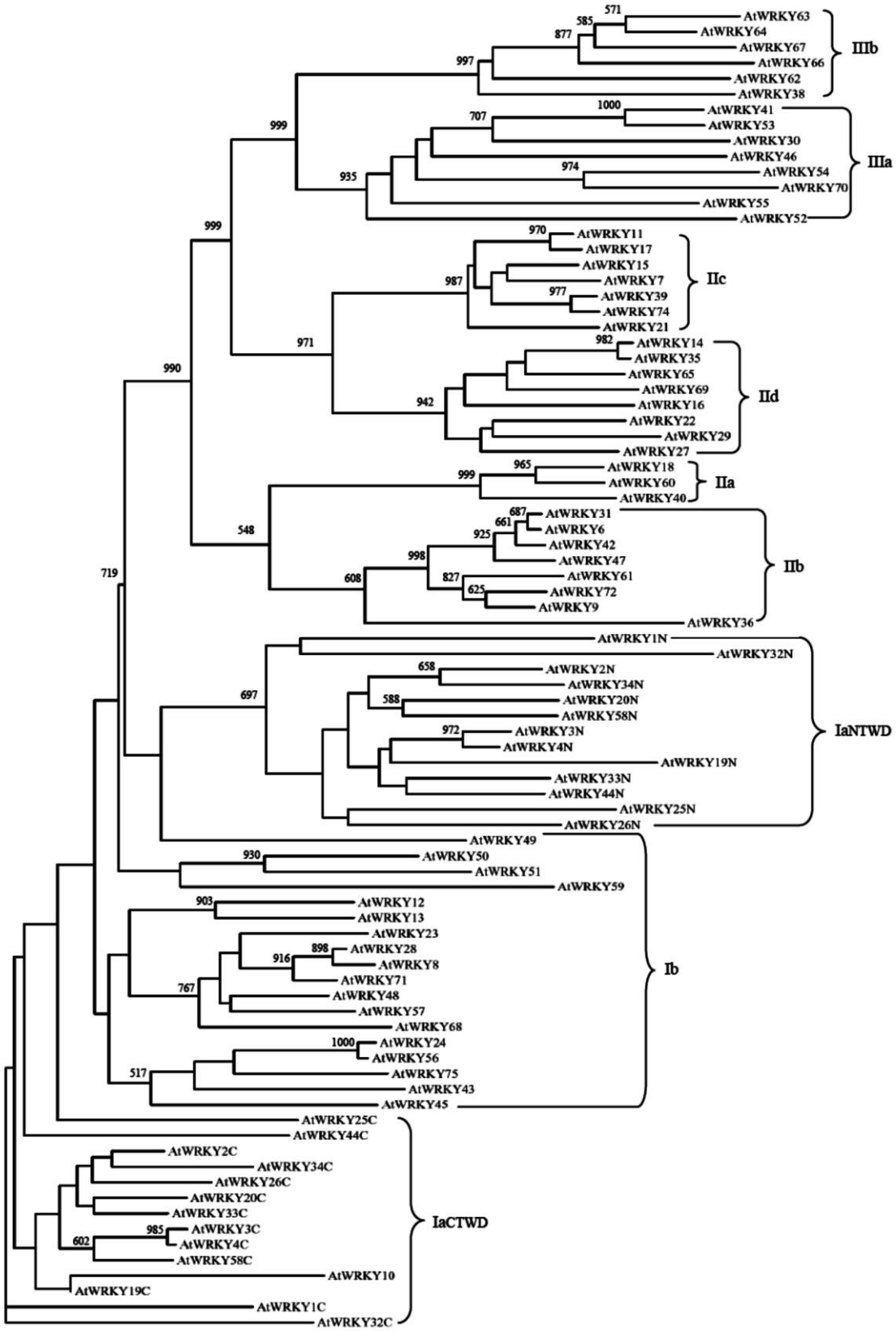


Figure 4. Phylogenetic tree of *Arabidopsis* WRKY domains based on amino acid sequence WRKY domain [67]. This figure is cited from the paper of Wu [67]. The late in numbers represent bootstrap values (≥500) based on 1000 replications. The members are grouped into groups (Ia-Ib, IIa-IIc, and IIIa-IIIb). *Arabidopsis* WRKY protein (AtWRKY) with the suffix -N or -C indicates the N-terminal (NTWD) or the C-terminal WRKY domain (CTWD) in one protein.

1.5.1.2 Origin and evolution

WRKY domains have been found in more than 40 plants species [64]. Fungi and animals do not have WRKY genes, so it is thought that WRKY is a plant specific transcription factor family of only higher plants [65]. However, the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) reports that WRKY-like sequences exist in both amoebozoa (*Dictyostelium discoideum*), diplomonads (*Giardia lamblia*) [68] and in green algae (*Chlamydomonas reinhardtii*). WRKY expressed sequence tags (ESTs) had been identified in lower plants, for example ferns (*Ceratopteris richardii*) and mosses (*Physcomitrella patens*) [68]. The number of WRKY transcription factor family members increases from lower to higher plants. At least 12 WRKY genes were identified in the genome of mosses (*Physcomitrella patens*) and 21 in cycases (*Cycas revolute*) [68,69]. Earlier studies found 74 WRKY transcription factor genes in *Arabidopsis* [68] (http://www.mpipz.mpg.de /20974/Arabidopsis_WRKY_Superfamily), whereas recent data from PlantTFDB 3.0 identified 90 WRKY transcription factors (72 loci) [58] (http://planttfdb.cbi.pku.edu.cn/family.php?sp=Ath&fam=WRKY). More than 100 WRKY transcription factors are present in the rice genome (*Oryza sativa*) [67,70,71], 59 in grapevine (*Vitis vinifera L*) [72], 86 in *Brachypodium distachyon* [73], and 119 WRKY genes in the *Zea maydis* variety B73 [74]. These results suggest that the WRKY transcription factor family may be derived from archaic and lower eukaryotic cells, and that the number of WRKY family members has increased during plant evolution for adaption of higher plants to different environments.

All of the WRKY transcription factors from the lower eukaryotes (amoebozoa, diplomonads, green algae, ferns and mosses), belong to group I. Fifteen out of 21 transcription factors from cycases also belong to group I, whereas the other 6 members belong to group II. From the evolutionary perspective subgroup Ia of the WRKY transcription factors is the most archaic and conserved group [75]. The group II members may be derived from subgroup Ia by the loss of one WRKY domain. The group III members may have evolved from group II by changing histidine (H) to cysteine (C) in the zinc finger motif. The group II transcription factor may replicate WRKY domain to evolve into subgroup Ib [75]. When comparing the sequences of the three WRKY transcription factor families, group I and II are much more conserved as group III [68].

1.5.1.3 Biological function

Like most of the transcription factors, WRKY transcription factors bind to specific target genes. It is demonstrated that they bind the (T)(T)TGAC(C/T) sequence motif, which is named as W-box [65]. The core W-box sequence is “TGAC” and most important for DNA binding. Mutations in any position of the “TGAC” core sequence abolish the binding by a WRKY transcription factor. The neighboring sequences of “TGAC” do not have a significant impact on DNA binding [76-79]. If a promoter region contains W-boxes, especially in tandem repeats, the gene is likely to be the target of WRKY transcription factors. The earliest WRKY transcription factor SPF1 discovered, plays a role in sucrose

signaling [63]. However, WRKY transcription factors are mainly involved in biotic and abiotic stress signaling pathways [66,80-82].

The expression profiles of 72 WRKY genes in *Arabidopsis* revealed that 49 genes are responsive to salicylic acid (SA) and pathogen treatment [83]. Fifteen out of 45 WRKY genes in rice are highly expressed upon inoculation of the fungal pathogen *Magnaporthe grisea* [84]. Thus, the main function of WRKY transcription factors is plant pathogen defense. In *Arabidopsis*, AtWRKY33 plays a positive role in pathogen defense against the fungal pathogen *Alternaria brassicicola* and *Botrytis cinerea* [85,86]. The single and double mutant of *Atwrky3* and *Atwrky4* are more susceptible than wild-type plants to infections with the fungal pathogen *Botrytis cinerea* and promote fungal growth. The AtWRKY4 overexpression line enhances plant susceptibility to the bacterial pathogen *Pseudomonas syringae* [87]. *Atwrky7*, *Atwrky11* and *Atwrky17* mutants are more susceptible to *Pseudomonas syringae* [88,89]. AtWRKY38, AtWRKY48 and WRKY62 function as negative regulators of plant resistance toward *Pseudomonas syringae* [90,91]. *Atwrky27* mutants show a delay in symptom development of the bacterial pathogen *Ralstonia solanacearum* [92]. In rice, three overexpression lines of OsWRKY13, OsWRKY71 and OsWRKY89, respectively are more resistant to bacterial blight (*Xanthomonas oryzae pv. Oryzae*) [93-95], and the three overexpression lines of OsWRKY13, OsWRKY53 and OsWRKY45 are more resistant to fungal blast (*Magnaportha grise*) [94,96-98]. Overexpression of the *VvWRKY1* gene in grapevine (*Vitis vinifera* L. cv. Cabernet Sauvignon) and tobacco enhances tolerance to the fungal pathogen downy mildew (*Plasmopara viticola*) [99,100] whereas overexpression of *VvWRKY2* in tobacco reduces susceptibility to the fungal pathogens (*Botrytis cinerea*, *Pythium spp* and *Alternaria tenuis*) [101]. HvWRKY1 and HvWRKY2 function as repressors of barley (*Hordeum vulgare*) defense to *Blumeria graminis* f sp. *hordei* [102].

WRKY transcription factors also play a role in regulating abiotic stress reactions. The expression profiles of *WRKY* genes in rice show that 54 *WRKY* genes were induced by cold, drought, salt stress or phytohormone treatment [71]. Overexpression of OsWRKY30 enhances drought tolerance in rice [103]. In *Arabidopsis*, AtWRKY33 is involved in salt stress and AtWRKY25 functions in both salt and heat stress [104,105]. WRKY transcription factor genes respond to abscisic acid (ABA) signaling, a phytohormone that mediates plant responses to abiotic stresses. *AtWRKY18*, *AtWRKY40*, and *AtWRKY60* genes are induced by ABA and negatively regulate ABA signalling [79,106,107].

Another important role of WRKY transcription factors is regulating plant development. TTG2 (TRANSPARENT TESTA GLABRA2)/AtWRKY44 is the first WRKY transcription factor, which was shown to be involved in plant development. AtWRKY44 forms a complex with R2R3 MYB and bHLH transcription factors and functions in trichome and seed coat development [108,109]. The *MINISEED3*（*MINI3*）gene encodes AtWRKY10 transcription factor and regulates seed development [110,111]. In addition, AtWRKY6 and AtWRKY75 are involved in *Arabidopsis*

responses to low- Phosphate (Pi) stress [112,113]. AtWRKY6 also function in boron deficiency and arsenate uptake [114,115]. AtWRKY6, AtWRKY53 and AtWRKY70 mediate leaf senescence in *Arabidopsis* [116-121].

1.5.1.4 Interaction of WRKY transcription factors with other proteins

In the past decade several studies have proven that WRKY transcription factors can interact with different kind of proteins [122]. One of the most important interactions is the WRKY-WRKY interaction. The *Arabidopsis* AtWRKY18, AtWRKY40 and AtWRKY60 proteins interact with each other and themselves [123]. AtWRKY30 was found to interact with AtWRKY53, AtWRKY54 and AtWRKY70 in a yeast-two-hybrid assay [124]. Very recently it was reported that AtWRKY13 interacts with itself, as well as with AtWRKY40 and AtWRKY57 in yeast [125]. The rice WRKYs, OsWRKY51 and OsWRKY71 interact with each other in a Bimolecular Fluorescence Complementation (BiFC) assay [126]. Some WRKYs were shown to interact with components of the MAPK (mitogen-activated protein kinases) signaling cascade [127]. For example AtWRKY25 and AtWRKY33 interact with MKS1 (MPK4 substrate 1) and AtWRKY33 becomes phosphorylated by AtMPK3/AtMPK6 [128,129]; the rice OsWRKY33 is phosphorylated by OsBWMK1 [130] and OsWRKY30 interacts with OsMPK3, OsMPK4, OsMPK7, OsMPK14, OsMPK20-4, whereas OsMPK20-5 is phosphorylated by OsMPK3, OsMPK7 and OsMPK14 [103]. In addition, WRKY transcription factors can interact with proteins containing a conserved FxxxVQxLTG or VQ motif, chromatin remodeling proteins, calmodulin, and other proteins [122].

1.5.2 ARF transcription factor family

Auxin response factors (ARF) regulate the expression of auxin responsive genes and play an important role in auxin signaling and plant development. ARF transcription factors bind to auxin response elements (AuxRE, TGTCTC) in auxin responsive promoters. The consensus sequence is TGTCTC, but the position 5 (T) and 6 (C) are not as important as the other positions for transcription factor binding [131,132]. However, double mutations at position 5 (T) and 6 (C) can impair the DNA binding affinity of some ARF transcription factors [131,132]. Nonetheless, the sequences TGTCNC or TGTCTN (N = A/G/C/T) are also considered as DNA binding elements for ARF transcription factors.

1.5.2.1 Protein structure

Most ARF transcription factors contain four important domains (Figure 5A). Domain I of the N-terminal end of the protein is the DNA binding domain (DBD), which is classified as a plant-specific B3-type [132,133]. Domain II in the middle region (MR) functions as an activation (AD) or repression domain (RD). Activation domains are rich in glutamine, serine and leucine residues whereas repression domains mainly contain serine, proline, leucine and glycine residues [134]. Domain III and IV are localized at the C-terminus of ARF proteins and are important for dimerization and interaction with ARF itself and auxin/indole acetic acid (Aux/IAA) proteins [132].

Arabidopsis contains 23 ARF proteins. ARF3, ARF13 and ARF17 lack domain III and IV, and ARF23 only has a part of domain I [134]. The middle region of ARF5, ARF6, ARF7, ARF8 and ARF19 are glutamine-rich (Q-rich) activation domains, and these ARF transcription factors function as transcriptional activators. In contrast, the other ARF transcription factors contain repression domains and function as repressors [134,135].

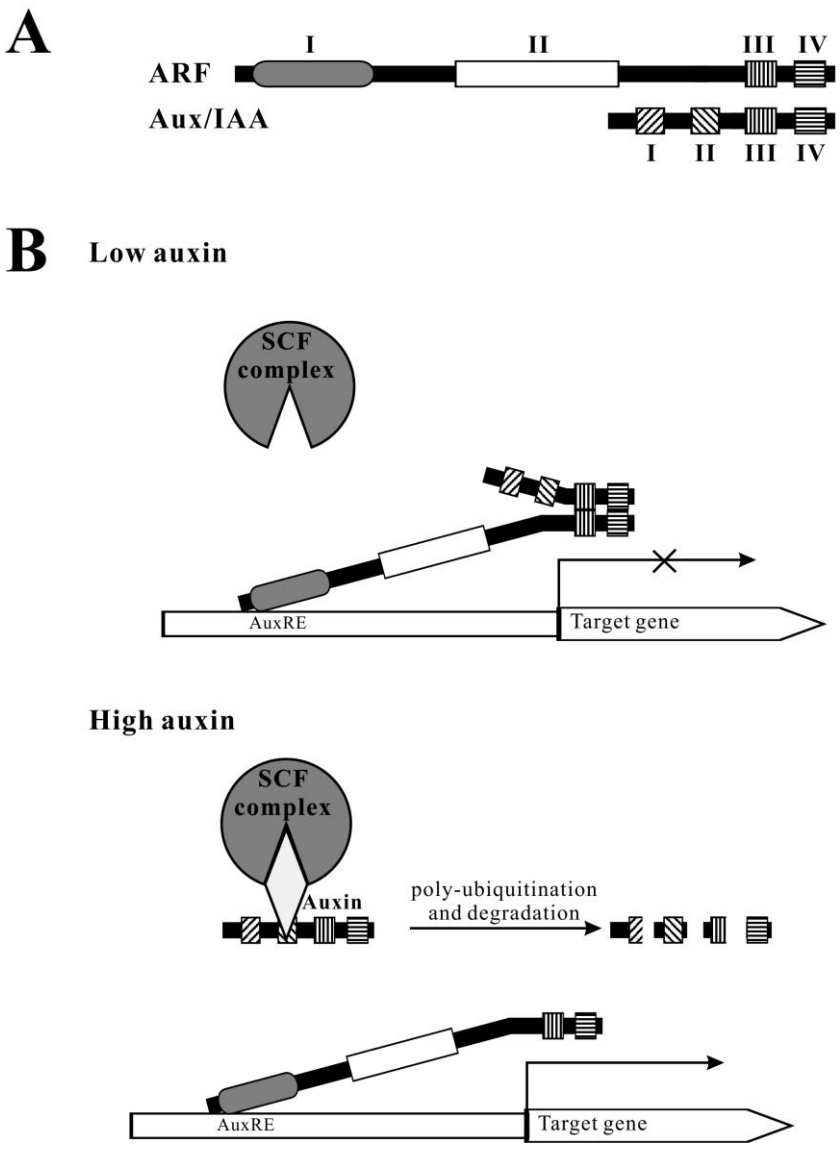


Figure 5. The structure of ARF and Aux/IAA proteins and model of auxin-dependent gene regulation.
(A) The structure of ARF transcription factors and Aux/IAA proteins. (B) Mode of regulation of target gene expression by ARF transcription factors which is blocked by interaction with Aux/IAA proteins at low auxin concentrations. High concentrations of auxins promote the poly-ubiquitination and degradation of Aux/IAA proteins, and then ARF transcription factors are released and activate target gene.

1.5.2.2 Biological function

ARF transcription factors play roles in many process of plant development, and their roles have been identified in studies on *arf* mutants by the classical forward genetic approaches. The *arf2* mutant is impaired in the development of normal rosette leaves, flowers and inflorescences [136]. The *arf3*

mutant develops an unusual gynoecium and shows a defect in floral patterning [137,138]. The *arf5/monopteros (mp)* mutant fails to form normal roots and cotyledons and has a dwarf growth phenotype [138,139]. The *arf7/nonphototropic hypocotyl 4 (nph4)* mutant is defective in hypocotyl tropisms and resistance to auxin and ethylene [138,140]. ARF8 transcription factor plays a role in uncoupling fruit development from fertilization and petal growth [141,142]. The phenotypes of the double mutants are usually much stronger than those of the single mutant, suggesting that related ARF transcription factors have somewhat redundant functions in *Arabidopsis* [134].

1.5.2.3 Aux/IAA proteins are repressors for the ARF transcription factors

Aux/IAA proteins are a kind of transcription regulators that interact with ARF transcription factors and function as repressors. There are 29 Aux/IAA genes in *Arabidopsis* [143]. The sizes of most Aux/IAA proteins are much smaller than ARF proteins, but Aux/IAA proteins also contain four domains, domain I, II, III and IV (Figure 5A). Domain I is the repression domain of Aux/IAA proteins [144,145]. Domain II is necessary and sufficient for interaction with the SCF (Skp, Cullin, F-box protein) ubiquitin ligase complex [146]. The sequences of domain III and IV of Aux/IAA proteins are similar to those of domain III and IV of the ARF transcription factors. The interaction between Aux/IAA proteins and ARF transcription factors is mediated via these two domains [131,147].

1.5.2.4 Auxin-dependent regulation of transcription

In *Arabidopsis*, ARF5, ARF6, ARF7, ARF8 and ARF19 transcription factors have been experimentally proven to function as activators in protoplasts [133,135,148]. These activators bind and activate auxin response genes in an auxin-dependent manner. The following model of auxin-dependent regulation has been established (Figure 5B) [149,150]: In low auxin conditions, Aux/IAA proteins interact with and inhibit ARF transcription factors via domain III and domain IV. The target genes containing AuxRE (TGTCTC) in their promoter regions are repressed. However, Aux/IAA proteins are unstable and short-lived proteins that are degraded via the SCF proteasome pathway. Auxin promotes and initiates this process by enhancing the interaction between TIR1 (transport inhibitor response 1) or AFB (auxin signaling F box) proteins and the Aux/IAA proteins. TIR1 and AFB proteins are F-box proteins that form the SCF ubiquitin ligase complex together with Skp (ASK1) and Cullin (CUL1). Aux/IAA proteins are poly-ubiquitinated by the SCF complex, and then degraded by the proteasome. The inhibition of ARF transcription activity is removed with the degradation of Aux/IAA proteins, and consequently binding of ARF to AuxRE containing promoter elements. However, it is still unclear how Aux/IAA proteins cooperate with ARF repressors or how ARF repressors cooperate with ARF activators. One opinion is that repression function of ARF repressors is independent of Aux/IAA protein and auxin and is due to competing with ARF activators for AuxRE binding elements in auxin response promoters [134].

1.6 Oncogene expression in plant cells

In the 1980s, the transcripts of the T-DNA-encoded oncogenes have been detected in transformed plant tissues. Thus, the T-DNA must contain promoter regions, at least the core promoter sequence elements for the transcription in the plant cell. In 1987 it was demonstrated that the 184 bp of the 5' non-coding region and 270 bp of the 3' non-coding region of the *Ipt* gene from the octopine Ti plasmid pTiAch5 were sufficient for its transcription [151]. Expression of the GUS reporter gene in transfected tobacco plants demonstrates that the 5' non-coding region of the *Ipt* gene had promoter activity and the sequences between −185 bp and −139 bp upstream of the translational start codon are essential to drive GUS expression [152]. A 30 bp sequences of this region, designated as *cyt-1*, function as binding element for some unknown proteins, designated CBF (*cyt-1* binding factor), from tobacco nuclear extracts [153]. Furthermore, the yeast DNA binding protein Multicopy Inhibitor of Galactose (MIG1) binds to the *cyt-1* element and the *Arabidopsis* AtSkp1 (*A. thaliana* S-phase kinase-associated protein 1 homolog) inactivates MIG1 binding [154]. The 5' non-coding region of the *Ipt* oncogene is likely to function as a promoter, at least as a core promoter. However, it is still unknown, which plant transcription factor regulates *Ipt* expression. In addition, the promoter regions of the *IaaH* and *IaaM* oncogenes are also not known.

1.7 Objectives of this study

This study focuses on regulation of expression of the agrobacterial oncogenes in a host plant. It is well known that the T-DNA region of Ti plasmid is transferred and integrated into host plant genome. In the host cells the oncogenes, *IaaH*, *IaaM* and *Ipt*, of T-DNA region are transcribed, and they are essential for biosynthesis of auxin and cytokinin, which are important for cell proliferation and development of the crown galls. However, the mechanism, how host plants regulate expression of these oncogenes is still unknown. The first sequenced *A. tumefaciens* genome was that of strain C58 and used in this study for infection of the model plant *Arabidopsis thaliana*. The first objective is to identify the oncogene promoters and *cis*-regulatory sequence motives required for their activity in the host cell. The second objective is identification of the host transcription factors which activate the oncogene promoters. The yeast-one-hybrid (Y-1-H) and protoplast transactivation (PTA) system are high-throughput methods for the screening of transcription factor libraries and were both applied in this study. The library for the Y-1-H assay contains approximately 1500 transcription factor cDNAs of *Arabidopsis thaliana* and was kindly provided by N. Mitsuda (Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan) [155]. The library used for the PTA system contains more than 400 coding sequences for transcription factors and was provided by the group of W. Dröge-Laser (Julius-von-Sachs-Institute, Pharmaceutical Biology, University of Wuerzburg, Germany) [156]. The transcript levels of potential candidate transcription factor genes will be quantified to prove their responsiveness to the agrobacterial pathogen. Mutant plants of the transcription factor candidates are used to prove their role in crown gall tumor

development. The effects of auxin and cytokinin on oncogenes expression will also be analyzed, because these phytohormones are essential for crown gall tumor development and pathogen defense. Finally, a working model about the regulation of oncogene expression in the host cell will be presented which summarizes the molecular mechanisms of how the expression of genes of prokaryotic origin is regulated in a eukaryotic cell. In other words, this study explains one aspect of how *A. tumefaciens* exploits the plant transcription machinery to support expression of its genes in the host cell to initiate crown gall tumor development, which then serves as an ecological niche for *A. tumefaciens*.

2. Material and methods

2.1 Cultivation of microorganism

2.1.1 Strains

Table 1 Microorganism strains

Strains	Microorganism	Genotype	Antibiotic resistance	Reference
C58	*Agrobacterium tumefaciens*	C58 oncogenic	–	[157]
GV3101	*Agrobacterium tumefaciens*	Non oncogenic, derivative of *Agrobacterium tumefaciens* C58, contains plasmid pMP90	Rifampicin Gentamicin	[158]
MRF	*Escherichia coli*	Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F′ proAB $lacI^qZ\Delta M15$ Tn10 (Tet^r)]	–	Stratagene
SoluBL21	*Escherichia coli*	F^- ompT $hsdS_B$ ($r_B^-m_B^-$) gal dcm (DE3)*	–	[159]
YM4271	Yeast	MATa, ura3-52, his3-200, lys2-01, ade2-101, ade5, trp1-901, leu2-3, 112, tyr1-501,gal4Δ, gal80Δ, ade5 : : hisG	–	[160]

*The SoluBL21 strain contains uncharacterized mutations obtained through special selection criteria. These mutations make the strain able to express insoluble proteins in soluble form, fully or partially, in most tests conducted.

2.1.2 Media

Table 2 Components of medium for microorganism

Mediums	Components (1 L)	Microorganism
LB	10 g tryptone; 5 g yeast extract; 10 g NaCl; 15 g agar (plate only)	*Escherichia coli*
SOC	20g tryptone; 5 g yeast extract; 0.5 g NaCl; 5 g $MgSO_4 \cdot 7H_2O$	*Escherichia coli*
SOB	SOC buffer, 20 mM glucose	*Escherichia coli*
YEB	5 g yeast extract; 5 g tryptone; 5 g sucrose; 50mM $MgSO_4$; 15 g agar (plate only)	*Agrobacterium tumefaciens*
KB	20 g protease peptone; 1.5 g K_2HPO_4; 0.87% glycerol; 600 μM $MgSO_4$; 15 g agar (plate only)	*Agrobacterium tumefaciens*
YPDA	20 g peptone; 10 g yeast extract; 20 g glucose; 0.03 g adenine hemisulfate; 20 g agar (plate only)	Yeast
SD	6.7 g yeast nitrogen base without amino acids; 20 g glucose; appropriate Dropout (Clontech, Otsu, Japan) ; 20 g agar (plate only)	Yeast

The LB, SOC, SOB, YEB and KB mediums are autoclaved at 121 ℃ for 15–20 min by standard program. YPDA and SD mediums are autoclaved at 121 ℃ for 15 min by rapid cooling program or autoclaved at 108 ℃ for 30 min. If YPDA and SD mediums are autoclaved at a higher temperature, for a longer period of time, or repeatedly, the glucose in the mediums will turn to darken and it will decrease the performance of the medium.

Table 3 List of antibiotics

Antibiotic	Stock Solution	*Escherichia coli*	*Agrobacterium tumefaciens*	Plant
Ampicillin	50 mg/mL in water	50 μg/mL	−	−
Kanamycin	50 mg/mL in water	50 μg/mL	100 μg/mL	100 μg/mL
Gentamicin	25 mg/mL in water	−	25 μg/mL	−
Rifampicin	10 mg/mL in DMSO	−	10 μg/mL	−
Spectinomycin	100 mg/mL in water	100 μg/mL	100 μg/mL	−
Hygromycin	40 mg/mL in water	40 μg/mL	−	30 μg/mL

2.1.3 Cultivation of *Agrobacterium tumefaciens*

Agrobacterium tumefaciens strains (2.1.1 Table 1) were stored in 25% (v/v) glycerol in YEP medium (2.1.2 Table 2) at −80 ℃. *A. tumefaciens* strains were streaked on YEP agar plates (2.1.2 Table 2) containing appropriate antibiotic (2.1.2 Table 3) and incubated at 28 ℃ for 2 days to obtain single colonies. Plates were stored at 4 ℃ no longer than one month. Overnight culture of *A. tumefaciens* was from colonies on the agar plates. Colonies were transferred into 3–5 mL YEB or KB liquid medium (2.1.2 Table 2) containing the appropriate antibiotic in 13 mL tubes with assembled two-position ventilation cap (with position 1: cap lightly pushed on, ventilated) (SARSTEDT, Nuembrecht, Germany) and incubated at 140 rpm in a rotary shaker at 28 ℃ for overnight.

2.1.4 Cultivation of *Escherichia coli*

Escherichia coli strains (2.1.1 Table 1) were stored in 17% (v/v) glycerol in LB medium (2.1.2 Table 2) at −80 ℃. *E. coli* strains were streaked or spread on LB agar plates (2.1.2 Table 2) containing the appropriate antibiotic (2.1.2 Table 3) and incubated at 37 ℃ for overnight to obtain single colonies. Plates were stored at 4 ℃ no longer than one month. Overnight culture of *E. coli* was from colonies on the agar plates or stock strains. Colonies or stock strains were transferred into 3–5 mL LB liquid medium (2.1.2 Table 2) containing the appropriate antibiotic in 15 mL centrifuge tubes with conical base (SARSTEDT, Nuembrecht, Germany) for small-scale culture or 100 mL LB liquid medium containing the appropriate antibiotic in 250 mL erlenmeyer flask for large-scale culture, and incubated at 150 rpm in a rotary shaker at 37 ℃ for overnight.

2.1.5 Cultivation of yeast

Yeast strains (2.1.1 Table 1) were stored in 25% (v/v) glycerol in YPDA medium (2.1.2 Table 2) at −80 ℃. YPDA strains were streaked or spread on YPDA agar plates or SD selection plates (2.1.2 Table 2), and incubated at 30 ℃ for 2–3 days to obtain single colonies. Plates were stored at 4 ℃ no longer than one month. Overnight culture of yeast was from colonies on the agar plate. Colonies were transferred into 3–5 mL YPDA liquid medium (2.1.2 Table 2) in 13 mL tubes with assembled two-position ventilation cap (with position 1: cap lightly pushed on, ventilated) (SARSTEDT, Nuembrecht,

Germany) for small-scale culture or 50 mL LB liquid medium 250 mL erlenmeyer flask for large-scale culture, and incubated at 140 rpm in a rotary shaker at 30 ℃ for overnight.

2.2 Manipulation of microorganism

2.2.1 Chemical competent *Escherichia coli*

Overnight cultures (2.1.4) were added to 100 mL SOB medium (2.1.2 Table 2) and cultivated at 37 ℃ in a rotary shaker at 220 rpm. The bacterial culture at an OD_{600} of 0.4–0.5 was transferred into 50 mL tubes, cooled on ice for 15 min and centrifuged at 2500 rcf and 4 ℃ for 15 min. The pellet was re-suspended in 15 mL sterile and ice cold TFB1 buffer [30 mM KAc; 100 mM RbCl; 10 mM $CaCl_2$; 50 mM $MnCl_2 \cdot 4H_2O$; 12% Glycerol; pH=5.8 (with acetic acid)] and incubated for 30 min on ice. Following a second centrifugation step for 10 min at 2500 rcf at 4 ℃ the pellet was re-suspended in 2 mL ice cold TFB2 buffer [10 mM Na-MOPS; 10 mM RbCl; 75 mM $CaCl_2$; 12% Glycerol; pH=6.8 (with NaOH)]. Aliquots of 50 µL were frozen in liquid nitrogen and stored at −80 ℃. Components of TFB1 and TFB2 buffer were sterilized using a 0.2 µm filter.

2.2.2 Transformation of *Escherichia coli*

The plasmids were transformed into *E. coli* strains using the heat shock method. The plasmid was added into competent *E. coli* cells and mix gently. The mixture was incubated on ice for 20–30 min and heat-shock the cells for 90 s at 42 ℃ in water bath without shaking, and then immediately transfer the tube to ice for incubation of 1–2 min. 500 µL room temperature SOC medium was added to the mixture and the bacteria was incubated at 37 ℃ for 0.5–1 h with shaking at 150 rpm. The bacteria were centrifuged 3000 rpm for 1 min, and the 400 µL supernatant was removed. Finally the pellet was re-suspended and 50–100 µL suspensions were spread on LB selection agar plates (2.1.2 Table 2) containing the appropriate antibiotic (2.1.2 Table 3) and incubated, up-side-down, at 37 ℃ for overnight.

2.2.3 Transformation of yeast

Several colonies, 2–3 mm in diameter were picked into 1 mL YPDA liquid medium (2.1.2 Table 2) and vortexed vigorously for 5 min to disperse any clumps. This 1 mL YPDA medium with yeast cells was transferred into a 250 mL erlenmeyer flask containing 50 mL YPDA medium and the culture was incubated at 30 ℃ for 16–18 h with shaking at 250 rpm to stationary phase (OD_{600} > 1.5). 10–30 mL overnight culture was transferred to a 1 L erlenmeyer flask containing 300 mL YPDA medium to get the OD_{600} up to 0.2–0.3. The yeast culture was incubated at 30 ℃ for 3 h with shaking (230 rpm). At this point, the OD_{600} should be 0.4–0.6. The yeast cells were centrifuged at 1000 rcf for 5 min at room temperature (20–21 ℃). The supernatants were discarded and the cell pellets were suspended in sterile water. The cells were pooled into one tube (final volume 25–50 mL) and centrifuged at 1000 rcf for 5 min at room temperature. The cell pellet was suspended in 1.5 mL freshly prepared and sterile 1×

TE/1× LiAc (10 mM Tris-HCl; 1 mM EDTA; pH 7.5; 100 mM LiAc). 0.1 µg plasmid DNA or 1 µg linearized plasmid DNA (for reporter vector integration) and 0.1 mg herring testes carrier DNA were added into 100 µL competent cells in a fresh 1.5 mL tube and mix by vortexing. The 600 µL freshly prepared and sterile PEG/LiAc solution (40% PEG 4000; 10 mM Tris-HCl; 1 mM EDTA; pH 7.5; 100 mM LiAc) was added to each tube and vortex at high speed for 10 s to mix. The mixture was incubated at 30 ℃ for 30 min with shaking at 200 rpm. 70 µL DMSO was added and mix well by gentle inversion (Do not vortex). The mixture was heat-shocked for 15 min in a 42 ℃ water bath, and then transferred on ice for 1–2 min. The cells were centrifuged for 30 s at 6000 rpm at room temperature. The supernatant was removed and the cell pellet was re-suspended in 0.5 mL sterile 1× TE buffer (10 mM Tris-HCl; 1 mM EDTA; pH 7.5). 100–500 µL suspended cells was spread on each SD selection agar plate (2.1.2 Table 2) and incubated, up-side-down, at 30 ℃ until colonies appear (generally, 2–4 days).

2.3 Cultivation of Plants

2.3.1 Cultivation of *Arabidopsis thaliana* on soil

Arabidopsis thaliana seeds were germinated on "sterile" soil (100 ℃ for 15 min) (Einheitserde Type P, Gebr. Hagera GmbH, Sinntal-Jossa, Germany) and separated into single pots with "sterile" soil as with two primary leaves (about 2 weeks). Plants were cultivated in growth chambers (Percival AR-66L2, Perry, USA) with approximately 120 µmol m^{-2} s^{-1} of fluorescent white light (TL70, Philips, Eindhoven, Netherlands and Osram 25 W, Osram, Munich, Germany) at 12 h light (22 ℃)/12 h dark (16 ℃) cycles at a relative humidity of 50–60%. *Arabidopsis thaliana* ecotype Columbia (Col-0) was used as wild type.

2.3.2 *Arabidopsis thaliana* mutants

Table 4 *Arabidopsis thaliana* mutants

Mutant	Gene locus	Accession numbers	Mutagen	Background	Insert position	Reference
wrky18	AT4G31800	GABI-Kat 328G03	T-DNA insertion	Col-0	Intron 2	[102]
wrky40	AT1G80840	SLAT_N40001	dSpm transposon insertion	Col-0	Intron 1	[102]
wrky60	AT2G25000	SALK_120706	T-DNA insertion	Col-0	Exon 2	[102]
tir1-1	AT3G62980	NASC ID: N3798	Ethyl methanesulfonate	Col-0	−	[161]

Double and triple mutants, *wrky18/40*, *wrky18/60*, *wrky40/60* and *wrky18/40/60* were generated from genetic crosses of single mutants. All of the *wrky* mutants were kindly provided by Dr. Imre E. Somssich (Department of Plant Microbe Interaction, Max Planck Institute for Plant Breeding Research, Cologne, Germany)

2.4 Manipulation of plants

2.4.1 Crown gall tumor induction and measurement

Overnight *A. tumefaciens* strain C58 (2.1.1 Table 1) cell cultures were grown in 5 mL KB liquid medium (2.1.2 Table 2) at 28 ℃, 140 rpm (2.1.3). *A. tumefaciens* cells were centrifuged at 8000 rpm for 1 min and suspended in 3 mL agromix buffer (0.01 M $MgCl_2$, 0.01 M MES pH 5.6). Suspended cells were cultured at 28 ℃, 140 rpm again for 2–3 h. OD_{600} was measured and adjusted to OD_{600} = 0.5. Young inflorescence stems (3 to 10 cm) of *Arabidopsis* were injected with 5 mL syringe for two times at one direction and two times at another perpendicular direction. One drop of *A. tumefaciens* cells was used for one injection. The pictures of crown gall tumors from different genotypes were taken by photomicrography Leica DFC500 (Leica, Wetzlar, Germany). Tumors were cut from the inflorescence stalk 25 days after inoculation with a scalpel and tweezer under a dissecting microscope Leica MZ6 (Leica, Wetzlar, Germany) and weighed in the balance.

2.4.2 *Arabidopsis thaliana* leaf infiltration

Overnight *A. tumefaciens* strain C58 (2.1.1 Table 1) cell cultures were grown in 5 mL KB liquid medium (2.1.2 Table 2) at 28 ℃, 140rpm (2.1.3). *A. tumefaciens* cells were centrifuged at 8000 rpm for 1 min and suspended in 3 mL agromix buffer with acetosyringone (0.01 M $MgCl_2$, 0.01 M MES pH 5. 6; 150 μM acetosyringone). Suspended cells were cultured at 28 ℃, 140rpm again for 2–3 h. OD_{600} was measured and adjusted to OD_{600} = 1.0. Leaves of 5 weeks *Arabidopsis* (Col-0) were infiltrated using 1 mL needleless syringe. The underside of the leaf was infiltrated until the whole leaf is turn into dark green.

2.5 Spectrophotometer measurements

Cuvette was used for cell culture (OD_{600}) measurements. Medium or buffer for cell culture was measured as blank. The concentrations of DNA, RNA and protein were quantified by NanoDrop 2000c UV-Vis Spectrophotometer (Thermo, Waltham, USA). Water or buffer which was used to dissolve DNA, RNA or protein was measured as blank.

2.6 DNA methods

2.6.1 Agarose gel electrophoresis

DNA samples in buffer (0.05% Bromophenol blue; 0.05% Xylen cyanol; 20 mM EDTA pH=8; 10% Glycerol) and RNA samples in buffer (47.5% Formamid; 0.0125% SDS; 0.0125% Bromophenol blue; 0.0125% Xylen cyanol; EDTA 0.25 mM pH=8; 10% Glycerol) were separated in agarose gels applying an electric field of 10–12 V/cm. The electrophoresis buffer used was 1 × TAE (40 mM Tris-base; 20mM acetic acid; 1 mM EDTA). DNA fragments shorter than 500 bp were separated in 2% agarose gel, longer fragments (500–1500 bp) in a 1% agarose gel. DNA fragments were stained by

addition of 0.005% GelGreen (Biotium, Hayward, USA). Size fractionated DNA bands were documented when excited with UV light of 260–360 nm applying an Image Master (VDS, Pharmacia, Uppsala, Sweden). As a size standard either Lambda PstI (Gibco/Invitrogen, Carlsbad, USA) or 100 bp marker (Biocat, Heidelberg, Germany) were used.

2.6.2 Plasmid extraction from *Escherichia coli*

Plasmid extraction was performed by alkaline lysis [162]. 3 mL overnight culture was centrifuged at 14500 rpm for 1min. The bacterial pellet was re-suspended in 50 µL TE buffer (10 mM Tris-HCl; 1 mM EDTA; pH 7.5). The bacteria were lysed in 400 µL TENS buffer (0.5% SDS; 0.1 M NaOH; 200 µg/mL RNAse; 10 mM Tris-HCl; 1 mM EDTA; pH=7.5) at room temperature. The alkaline lysate was neutralized by the addition of 200 µL 3 M NaAc (pH=5.2), which resulted in renaturation of the plasmid DNA but not the chromosomal DNA and precipitation of SDS and proteins. Precipitated cell debris, chromosomal DNA, SDS and proteins were removed by centrifugation at 10,000 rcf for 10 min. The supernatant was transferred into a 1.5 mL tube. The plasmid DNA was precipitated after addition of 800 µL isopropanol and centrifugation at 10,000 rcf for 5 min. The supernatant was discarded and the precipitate was washed with 500 µL 70% ethanol. The dry plasmid DNA was dissolved in 50 µL water and stored at −20 °C.

Plasmid for sequencing was extracted from 3 mL overnight culture by QIAGEN Plasmid Mini Kit (Qiagen, Hilden, Germany). Plasmid for protoplast transformation was extracted from 100 mL overnight culture by QIAGEN Plasmid Midi Kit (Qiagen, Hilden, Germany). The concentration of plasmid DNA was quantified by NanoDrop 2000c UV-Vis Spectrophotometer (Thermo, Waltham, USA).

2.6.3 DNA purification

DNA fragments, separated by agarose gel electrophoresis (2.6.1), were excised with a scalpel under UV light and purified by a silica column-based method. The QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) was used according to the manufacture ́s protocol. The DNA was eluted with water and stored at −20 °C. PCR products were directly purified according to the protocol of QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). The DNA was eluted with water and stored at −20 °C. The concentration of DNA was quantified by NanoDrop 2000c UV-Vis Spectrophotometer (Thermo, Waltham, USA).

2.6.4 Restriction enzyme digestion

Type II restriction enzymes were used to cut the DNA within their recognition sequence and create either blunt or sticky ends. Recombinant plasmids were analyzed by digestion with restriction enzymes to verify the successful insertion and the orientation of the fragment of interest. In general 1 µg DNA was digested with 1 unit of the respective endonuclease in the buffer system suggested by the

supplier (Fermentas, Thermo, Waltham, USA) for one hour at appropriate temperature, usually at 37 ℃. The digested DNA was analyzed by agarose gel electrophoresis (2.6.1).

2.6.5 DNA sequencing and sequence analysis

All sequencing reactions were based on the dideoxy chain termination method according to Sanger [163] and were performed by GATC Biotech (GATC Biotech, Constance, Germany). Sequence data was analyzed by using the DNAMAN Version 6 (Lynnon Corporation, Quebec, Canada).

2.6.6 Polymerase Chain Reaction (PCR)

A standard PCR reaction (shown as following) was prepared on ice in 0.5 mL tubes. The reaction mix was placed in thermal cycler (Mastercycler personal, Eppendorf, Hamburg, Germany). DreamTaq DNA Polymerase (Fermentas, Thermo, Waltham, USA) was used to produce PCR product with a 3´ adenylate overhang for TA cloning. The recombinant Phusion Cx Polymerase was used to produce blunt end and high fidelity PCR product. PCR products were analyzed by agarose gel electrophoresis (2.6.1).

Standard PCR reaction (50 µL volume):

Template	10–100 ng
5 × Buffer	10 µL
Forward primer (10 µM)	1 µL
Reverse primer (10 µM)	1 µL
dNTP (10 mM)	1 µL
Polymerase	0.5–1 µL
H_2O	to a final volume of 50 µL

Standard PCR program (for Phusion Cx):

Step 1: 98 ℃	1 min
Step 2: 98 ℃	20 s
Step 3: 50–60 ℃	25 s
Step 4: 72 ℃	15 s per kb
Step 5: repeat step 2 −4 for 25–40 cycles	
Step 6: 72 ℃	2–5 min
Step 7: 4 ℃	Store

2.6.6.1 Colony Polymerase Chain Reaction (PCR)

Colony PCR is a convenient high-throughput method for determining plasmid inserts directly from *E. coli* colonies. Each single colony was picked by white pipette tip into numbered 0.5 mL tubes containing 50 µL sterile water and mix well by pipetting. Each 10 µL mixture was transferred into

another new 0.5 mL tubes with the corresponding number and heat at 95–100 ℃ for 5–10 min for bacteria cell breakage. After heating step, the tubes were put on ice for template of colony PCR reaction.

Colony PCR reaction (20 µL volume):

Template	10 µL
5 × Buffer	4 µL
Forward primer (10 µM)	0.4 µL
Reverse primer (10 µM)	0.4 µL
dNTP (10 mM)	0.4 µL
Polymerase	0.3 µL
H_2O	4.5 µL

The colony PCR program was the same with standard PCR program (2.6.6) and PCR products were analyzed by agarose gel electrophoresis (2.6.1).

2.6.7 Quantitative real-time PCR (qRT-PCR)

Quantitative real-time PCR allows relative quantification of cDNAs synthesized from transcripts. This method bases on the detection and quantification of a fluorescent reporter, the signal of which is directly proportional to the amount of PCR product. Threshold cycle (Ct) is the amplification cycle at which the PCR product increases exponentially and is dependent on the amount of input cDNA. Ct of a sample is determined and compared with that of an external standard. As external standard serves a PCR product of the cDNA of interest of which the concentration is known and a calibration curve is prepared. The concentration of cDNA in an unknown sample is calculated by comparing the unknown to a set of standard samples of known concentration.

In order to normalize the amount of input cDNA for different samples the constitutively expressed housekeeping genes, *actin2* and *actin8* (*ACT2/8*) were determined in each sample as internal standard.

Components of qRT-PCR were mixed and pipetted into glass capillaries (Roche, Basel, Switzerland) or 96 wells PCR plate (Eppendorf, Hamburg, Germany). The capillaries were placed in the Lightcycler 3.1 (Roche, Basel, Switzerland) and 96 wells PCR plate was placed in the Mastercycler ep realplex (Eppendorf, Hamburg, Germany). The PCR was performed according to the protocol. The specificity of PCR products was confirmed by determining the temperature at which the PCR product is melting (melting point).

Primer-Mix (PM) (400 µL):

PM 6: 3 µL forward and 3 µL reverse primer stock (100 µM) in 394 µL HPLC H_2O;

PM 12: 6 µL forward and 6 µL reverse primer stock (100 µM) in 388 µL HPLC H_2O;

PM 18: 9 µL forward and 9 µL reverse primer stock (100 µM) in 382 µL HPLC H_2O.

Quantitative real-time PCR reaction mixture (20 µL volume):

ABSOLUTE™ QPCR SYBR® GREEN MIXES*	10 µL
Primer-Mix (PM)	8 µL
cDNA diluted 20 fold in tRNA-H_2O**	2 µL

* ABSOLUTE™ QPCR SYBR® GREEN MIXES (Thermo Fisher Scientific, Waltham, USA)

**tRNA-H_2O: 1 µL tRNA (10 µg/µL) (Sigma, St. Louis, USA) in 1 mL HPLC H_2O.

Standard quantitative real-time PCR program:

Denaturation:	95 °C	15 min
Amplification:	95 °C	15 s
	Tm °C	20 s
	72 °C	20 s
	79 °C	5 s
Repeat amplification step 40 cycles.		
Melting Curve	95 °C	5 s
	75 °C	30 s
	95 °C	0s increase from 75 °C to 95 °C slowly (0.3 °C/s)
Cooling	40 °C	30 s

2.6.7.1 Calibration curve for the external standard

Fragments, going to be quantified by real-time PCR, were used as external standards. Therefore, PCR products were purified and quantified by NanoDrop 2000c UV-Vis Spectrophotometer (2.6.3) (Thermo, Waltham, USA). A set of standards of known concentration was prepared by a 10 folds serial dilution from 20 fg to 0.02 fg of the purified PCR product to create a calibration curve. Specificity of the PCR products was confirmed by melting point analysis and agarose gel electrophoresis.

Standard series:

Standard 1 = 10 ng/µL	
Standard 3 = 100 pg/µL	(2 µL standard 1 in 198 µL HPLC H_2O)
Standard 5 = 1 pg/µL	(2 µL standard 3 in 198 µL HPLC H_2O)
Standard 7 = 10 fg/µL	(2 µL standard 5 in 198 µL tRNA-H_2O)
Standard 8 = 1 fg/µL	(20 µL standard 7 in 180 µL tRNA-H_2O)
Standard 9 = 0.1 fg/µL	(20 µL standard 8 in 180 µL tRNA-H_2O)
Standard 10 = 0.01 fg/µL	(20 µL standard 9 in 180 µL tRNA-H_2O)

2 µL standard 7 (20 fg), 8 (2 fg), 9 (0.2 fg) and 10 (0.02 fg) were measured for creation of the standard straight within the qRT-PCR as external reference. The detection (Ct) of standard 7 has to come along between 18 and 19. If the standards 7–10 can be measured in intervals of ~ 3.5 cycles and the regression error appears under 0.1, the standard row will be corrected.

2.6.7.2 Calculation of transcript numbers

Quantification of the fluorescence intensity data was carried out with the Lightcycler software (Roche, Basel, Germany) or realplex software (Eppendorf, Hamburg, Germany). The software calculates the amount of input cDNA according to the known amount of DNA of the calibration curve. Therefore the fluorescence intensity ($\log_{10}$) vs. cycle number of log-linear range of the PCR was calculated for both, the internal standard *ACT2/8* and the cDNA of interest. For normalization of the input cDNA the number of transcript molecules of interest was determined in relation to 10,000 *ACT2/8* transcripts for each. Assuming that 4.78 fg *ACT2/8* correspond to 10,000 *ACT2/8* molecules, the number of *ACT2/8* transcripts x could be calculated as follows:

$$number\ of\ ACT2/8\ transcripts = 10000\frac{m(x)}{4.78}$$

m (x): measured *ACT2/8* cDNA amount (fg).

The number of cDNA molecules of target gene was calculated as follows:

$$number\ of\ target\ gene\ transcripts = 10000\frac{435 * m(y)}{length(y) * 4.78}$$

435: the length of *ACT2/8* PCR product (bp)
length (y): the length of target gene PCR product (bp)
m(y): measured target gene cDNA amount (fg).

The normalized number of target gene relative to 10,000 molecules of *ACTIN2/8* was calculated as follows:

$$\text{number of target gene relative to 10,000 molecules of } ACT2/8 = 10000\frac{number\ of\ target\ gene\ transcripts}{number\ of\ ACT2/8\ transcripts}$$

2.6.7.3 Primers used in qRT-PCR

Primers used in qRT-PCR were designed by LightCycler Probe Design software version 1.0 (Roche, Basel, Germany) and synthesized by TIB MOLBIOL (TIB MOLBIOL, Berlin, Germany).

Table 5 Primers used in qRT-PCR.

gene name (Accession No.)	primer	Primer sequence (5' ➔ 3')	Size of product
ACTIN2/8 (ACT2, AT3G18780, ACT8, AT1G49240)	forward	GGTGATGGTGTGTCT	435 bp
	reverse	ACTGAGCACAATGTTAC	
IaaH (pTiC58, AE007871)	forward	ACCTTGATGCTGATGTGGCC	597 bp
	reverse	CCCCGATTGCTAACAGACG	
IaaM (pTiC58, AE007871)	forward	TGCCCAGCATCTAGTT	309 bp
	reverse	CAAGAGTGTTCGAGAGG	
Ipt (pTiC58, AE007871)	forward	CAGTTATTGGAGTGCG	290 bp
	reverse	TCCCATGAATCAACTTAT	
WRKY18 (AT4G31800)	forward	AAAGTTTCGACTGTCT	325 bp
	reverse	CCAACGCTAGTCTATGA	
WRKY40 (AT1G80840)	forward	TGACTAGAGACAATCCAT	413 bp
	reverse	ATACAATTTTCCGGTAAC	
WRKY60 (AT2G25000)	forward	CCAACGATAAAGCGAC	318 bp
	reverse	TTTCCTCAACTGGTTC	
ARF5 (AT1G19850)	forward	GATAAACCTACTCGG	424 bp
	reverse	GGCCACTCGTATTAGA	
ARR1 (AT3G16857)	forward	AACTCGTTTATGACGG	337 bp
	reverse	TTCGATTACCCATAGG	
ARR4 (AT1G10470)	forward	TCCTGCAAAGTTACGG	247 bp
	reverse	CCTCAAGGCATCTGTC	

2.7 RNA methods

2.7.1 Poly-A-mRNA extraction

Poly-A-mRNA was isolated by the oligonucleotide Poly-dTTP (25) bound to polystyrene beads (Dynabeads Oligo-[dT] 25, Invitrogen, Carlsbad, USA). The plant tissue was frozen in liquid nitrogen and homogenized in a ball mill (MM2000, Retsch, Haan, Germany). For 50 mg tissue was frozen in liquid nitrogen and homogenized in a ball mill (MM2000, Retsch, Haan, Germany), and then 1 mL lysis buffer was added. The lysate was centrifuged for 30 s at 10,000 rcf. The supernatant was incubated for 30 min with 20 µL Dynabeads at room temperature in an overhead rotor. The washing steps were performed according to the manufacturer's protocol. In order to exclude DNA contaminations, mRNA was eluted from the Dynabeads by incubation at 65 ℃ for 2 min. In a second step the eluted mRNA was again bound to the Dynabeads for 20 min at room temperature in an overhead shaker. After two washing steps with washing buffer without lithium dodecyl sulfate, the mRNA was eluted from the magnetic beads with 16 µL RNase-free water at 65 ℃ for 2 min. The concentration of mRNA was quantified by NanoDrop 2000c UV-Vis Spectrophotometer (Thermo, Waltham, USA). The mRNA was directly used for first-strand cDNA synthesis for 5' RACE assay or stored at −80 ℃.

2.7.2 Total RNA extraction from plant tissue

Approximate 50 mg plant tissue was frozen in liquid nitrogen and homogenized in a ball mill (MM2000, Retsch, Haan, Germany). The plant tissues were re-suspended in 450 µL buffer RLT with 10 µL/mL β-mercaptoethanol (β-ME) (RNeasy Plant Mini Kit, Qiagen, Hilden, Germany). The protocol for purification of total RNA from plant cells (RNeasy Plant Mini Kit, Qiagen, Hilden, Germany) and for on-column DNase digestion (RNase-Free DNase Set, Qiagen, Hilden, Germany) was performed. Total RNA was eluted by RNase-free water. The concentration of total RNA was quantified by NanoDrop 2000c UV-Vis Spectrophotometer (Thermo, Waltham, USA) and analyzed in 1% agarose gel (2.6.1). The total RNA was directly used for first-strand cDNA synthesis or stored at −80 ℃.

2.7.3 Total RNA extraction from *Agrobacterium tumefaciens*

Overnight *A. tumefaciens* strain C58 (2.1.1 Table 1) cell cultures were grown in KB liquid medium (2.1.2, Table 2) at 28 ℃, 140 rpm. 100 µM acetosyringone was used to induce the virulence of *A. tumefaciens*. 1.5 mL overnight culture was used for total RNA extraction. *A. tumefaciens* cells were washed by sterile water and re-suspended in 450 µL buffer RLT with 10 µL/mL β-mercaptoethanol (β-ME) (RNeasy Plant Mini Kit, Qiagen, Hilden, Germany). The suspended cells were transferred into LYSING MATRIX E tubes (MP Biomedicals, Solon, USA) and lysed with FastPrep-24 Instrument (MP Biomedicals, Solon, USA) for 30 s at speed 5.0 m/s. After centrifuge at 14500 rpm for 10 min, the supernatants were transferred into QIAshredder spin column (RNeasy Plant Mini Kit, Qiagen, Hilden, Germany), and then the protocol for purification of total RNA from plant cells (RNeasy Plant Mini Kit, Qiagen, Hilden, Germany) and for on-column DNase digestion (RNase-Free DNase Set, Qiagen, Hilden, Germany) was performed. Total RNA was eluted by RNase-free water. The concentration of total RNA was quantified by NanoDrop 2000c UV-Vis Spectrophotometer (Thermo, Waltham, USA) and analyzed in 1% agarose gel (2.6.1). The total RNA was directly used for first-strand cDNA synthesis or stored at −80 ℃.

2.7.4 First strand cDNA synthesis

Approximate 500–1000 ng total RNA was digested by DNase I (Fermentas, Thermo, Waltham, USA) for 30 min at 37 ℃. 25 mM EDTA was added and the mixture was incubated at 70 ℃ for 10 min for termination of DNase I digestion. First strand cDNA synthesis by Oligo(dT)18 primer or random hexamer primer (Fermentas, Thermo, Waltham, USA) was performed by the following protocol.

First strand cDNA synthesis reaction (20 μL volume):

Mixture after DNase I digestion	11 μL
dNTP (10 mM)	2 μL
Oligo(dT)18 primer or random hexamer primer 100 μM	1 μL
5 × Buffer	4 μL
RevertAid M-MuLV Reverse Transcriptase (200 U/ μL)	1 μL
RiboLock RNase Inhibitor (20 U/μL)	1 μL

First strand cDNA synthesis by Oligo(dT)18 primer program:

42 ℃	1–1.5 h
70 ℃	5 min

First strand cDNA synthesis by random hexamer primer program:

25 ℃	5 min
42 ℃	1–1.5 h
70 ℃	5 min

The cDNA was diluted at 1:20 in tRNA-H_2O and used as template for standard PCR or qRT-PCR reaction.

2.7.5 5'- rapid amplification of cDNA ends (5' RACE) assay

mRNA from crown gall tumor callus was used for analyzing transcription start sites of the oncogenes in plant cell. mRNA was extracted by Dynabeads Oligo $(dT)_{25}$ (Invitrogen, Carlsbad, USA) from approximate 50 mg tumor callus (2.7.1). 5' ends of the oncogenes cDNA were amplified by SMARTer™ RACE cDNA Amplification Kit (Clontech, Otsu, Japan). Briefly, first-Strand cDNA was generated by SMARTScribe™ Reverse Transcriptase and primed using SMARTer II A Oligonucleotide and 5'-CDS Primer A. The fragments of 5' ends of the oncogenes cDNA were amplified by DreamTaq DNA Polymerase (Fermentas, Thermo, Waltham, USA) and primed using Universal Primer A Mix (UPM) and specific target primer (2.6.6). The PCR products were inserted into pGEM-T Easy Vector (Promega, Fitchburg, USA) by TA cloning (2.8.2) and the resulting recombinant vectors were transformed into MRF' *E. coli* strain (Table 1) (2.2.2). More than three independent colonies were sequenced for analyzing transcription start sites.

2.8 Cloning Strategies

2.8.1 Enzyme digestion and ligation cloning

DNA fragments and target vectors were digested by Type II restriction enzymes (Fermentas, Thermo, Waltham, USA) at 37 ℃ for 4–6 h or overnight (2.6.4). After digestion, DNA fragments were purified

by QIAquick PCR Purification Kit or QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) (2.6.3) and vectors were separated by agarose gel electrophoresis (2.6.1) and the linearized vectors were excised with a scalpel under UV light and purified by QIAquick Gel Extraction Kit protocol (Qiagen, Hilden, Germany) (2.6.3). The digested DNA fragments and linearized vectors were quantified by NanoDrop 2000c UV-Vis Spectrophotometer (Thermo, Waltham, USA) and mixed at a molar ratio from 3∶1 to 6∶1 (DNA fragment ∶ linearized vectors). The ligation reaction was performed by T4 DNA ligase (Fermentas, Thermo, Waltham, USA) at 22 ℃ for 3 h or at 16 ℃ overnight. The recombinant plasmids were transformed into MRF' *E. coli* strain (2.1.1 Table 1) (2.2.2). The *E. coli* cells were spread on LB on selection agar plates (2.1.2 Table 2) containing appropriate antibiotics (2.1.2 Table 3) and incubated at 37 ℃ overnight. Positive colonies were determined by colony PCR (2.6.6.1).

2.8.2 TA cloning

DNA fragments were firstly amplified by Phusion polymerase and blunt-ended PCR products were purified by QIAquick PCR Purification Kit (Qiagen Hilden, Germany) (2.6.3). An A-tailing procedure for blunt-ended PCR fragments was performed by an elongation step with DreamTaq polymerase (Fermentas, Thermo, Waltham, USA) at 72 ℃ for 10 min. 3´A-tailed fragments were purified by QIAquick PCR Purification Kit (Qiagen Hilden, Germany) (2.6.3) and TA cloning into pGEM-T Easy Vector (Promega, Fitchburg, USA) was performed with half dose according to the manufacture's protocol. The recombinant plasmids were transformed into MRF' *E. coli* strain (2.1.1 Table 1) (2.2.2). The *E. coli* cells were spread on LB selection agar plates (2.1.2 Table 2) containing appropriate antibiotics (2.1.2 Table 3) and incubated at 37 ℃ overnight. Positive colonies were determined by colony PCR (2.6.6.1).

2.8.3 Uracil-specific excision reagent (USER) cloning

Uracil excision-based cloning procedure was performed as described by Nour-Eldin et al., [164]. A total of 5 μg the respective plasmid DNA was digested with 40 units PacI restriction enzyme (New England Biolabs, Ipswich, USA) in a total volume of 200 μL at 37 ℃ overnight. The following day additional 20 units of PacI were added together with 20 units of Nt.BbvCI (New England Biolabs, Ipswich, USA) and the reaction mixture was incubated at 37 ℃ for 2 h. The generated a linearized USER vector with 3´overhangs was finally purified by QIAquick PCR Purification Kit (Qiagen Hilden, Germany) (2.6.3).

The sequences of primers used to amplify target DNA fragments for USER vectors were shown as following.

Forward primer: 5' GGCTTAAU + target DNA specific sequence 3'
Reverse primer: 5' GGTTTAAU + target DNA specific sequence 3'
Reverse primer for C-terminal fusion protein: 5' GGTTTAAU + CC + target DNA specific sequence without stop codon 3'

DNA fragments were amplified by Phusion Cx polymerase using USER primers (2.6.6). The PCR products were mixed with the linearized USER vectors and USER enzyme (New England Biolabs, Ipswich, USA) as following.

USER cloning reaction (10 µL volume):

Linearized USER vector	1.5 µL
USER enzyme	1 µL
PCR product	3–5 µL
TE	to a final volume of 50 µL

The USER cloning reaction was incubated at 37 ℃ for 20 min and then at 25 ℃ for 20 min. The recombinant plasmids were transformed into MRF' *E. coli* strain (2.1.1 Table 1) (2.2.2). The *E. coli* cells were spread on LB selection agar plates (2.1.2 Table 2) containing appropriate antibiotics (2.1.2 Table 3) and incubated at 37 ℃ overnight. Positive colonies were determined by colony PCR (2.6.6.1).

2.9 Protein methods

2.9.1 Expression of recombinant proteins in *Escherichia coli*

The full length of WRKY40 coding sequence (CDS) including stop codon was cloned into pET28b at Nde I and Xho I restriction enzymes sites to expressed His-tag N-terminal fusion protein (2.8.1). The recombinant plasmid was transformed into SoluBL21 *E. coli* strain (2.1.1 Table 1) (2.2.2). The SoluBL21 cells were spread on LB selection agar plates (2.1.2 Table 2) containing kanamycin (2.1.2 Table 3) and incubated at 37 ℃ overnight. Single colony was picked into 3–5 mL LB liquid medium (2.1.2 Table 2) containing kanamycin (2.1.2 Table 3) and incubated at 150 rpm in a rotary shaker at 37 ℃ overnight. 100 µL overnight cultures were transferred into 100 mL LB liquid medium containing kanamycin in 500 mL erlenmeyer flask and incubated 150 rpm in a rotary shaker at 37 ℃ for 2–3 h. The bacterial cultures were induced by adding 0.5 mM IPTG at OD_{600} = 0.6 and incubated at 16 ℃ and 150 rpm overnight. The overnight culture was centrifuged at 4000 rpm and 4 ℃ for 15 min. The bacteria pellets were washed by 20 mL 1× Ni-NTA binding buffer (300 mM NaCl; 20 mM Tris-Cl pH=8.0; 10 mM imidazole) and centrifuged at 4000 rpm and 4 ℃ for 15 min. The bacteria pellets were directly for purification or stored at −20 ℃.

2.9.2 Purification of recombinant proteins from *Escherichia coli*

Bacteria pellets (2.9.1) were re-suspended in 25 mL precooled 1× Ni-NTA binding buffer (300 mM NaCl; 20 mM Tris-Cl pH=8.0; 10 mM imidazole) containing cOmplete Protease Inhibitor Cocktail Tablets (Roche, Basel, Switzerland) in 50 mL tube. The suspended bacteria were sonicated on ice water mixture by sonicator (sonopuls HD3200, Bandelin, Berlin, Germany) for 5–15 min until the solution was clarified. The sonication program was 10 s bursts at 50 W with a 15–30 s cooling period

between each burst. The clarified extracts were centrifuged at 13000 rpm and 4 ℃ for 15 min. The supernatants were transferred into new tubes and centrifuged at 13000 rpm and 4 ℃ for another 15 min. The supernatants were used for Ni-NTA His•Bind Resin (Novagen, Merck Millipore, Darmstadt, Germany) purification. 0.5 mL Ni-NTA His•Bind Resin were washed by 1 mL 1× Ni-NTA binding buffer for three times and mixed with the supernatants of cell extracts on the rotor at 4 ℃ for 1 h. The mixture was loaded on the purification column and the residual liquid was drained by gravity. 5 mL 1× Ni-NTA binding buffer (300 mM NaCl; 20 mM Tris-Cl pH=8.0; 10 mM imidazole) washed the column. 30 mL 1× Ni-NTA washing buffer (300 mM NaCl; 20 mM Tris-Cl pH=8.0; 40 mM imidazole) washed the column. Finally, 5 mL 1× Ni-NTA elution buffer (300 mM NaCl; 20 mM Tris-Cl pH=8.0; 300 mM imidazole) were used to elute the His-tag recombinant protein. The eluted solution was buffer-exchanged with PBS buffer (137 mM NaCl; 2.7 mM KCl; 10 mM Na2HPO4; 2 mM KH_2PO_4; pH=7.4) by PD-10 Desalting Columns (GE Healthcare Life Sciences, Little Chalfont, United Kingdom) according to spin protocol. The recombinant proteins in PBS buffer were used for Electrophoretic Mobility Shift Assays (EMSA).

2.9.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used for the separation of proteins. Monomeric acrylamide polymerize was polymerized into polyacrylamide chains by the addition of ammonium persulfate (APS) and tetramethylenediamine (TEMED). The 10% polyacrylamide separating gel (10 mL per gel) was prepared with Rotiphorese Unit PROclamp MINI set (Roth, Karlsruhe, Germany) as following.

4 × separating gel buffer (1.5 M Tris-Cl, pH=8.8; 0.4% SDS)	2.5 mL
H_2O	4.9 mL
Rotiphorese Gel 40 (29:1) (Roth, Karlsruhe, Germany)	2.5 mL
10% ammonium persulfate (APS)	50 µL
TEMED (Roth, Karlsruhe, Germany)	5 µL

The water was overlaid on top of the separating (lower) gel. After polymerization (30–45 min) the water was removed and the 3.2% stacking (upper) gel was poured on top of the separating (lower) gel with comb. The 3.2% polyacrylamide stacking gel (5 mL per gel) was prepared as following.

4 × stacking gel buffer (0.5 M Tris-Cl, pH=6.8; 0.4% SDS)	1.25 mL
H_2O	3.3 mL
Rotiphorese Gel 40 (29:1) (Roth, Karlsruhe, Germany)	0.4 mL
10% ammonium persulfate (APS)	50 µL
TEMED (Roth, Karlsruhe, Germany)	5 µL

SDS-PAGE loading buffer (0.45 M Tris-Cl, pH=6.8; 10% glycerol; 1% SDS; 0.01% bromophenol blue; 0.05 M DTT) was added to the protein samples, followed by denaturation at 100 ℃ for 5 min. Electrophoresis was performed vertically in an electrophoresis tank (Rotiphorese Unit PROclamp MINI set, Roth, Karlsruhe, Germany) with electrophoresis buffer (0.1 M Tris base; 0.384 M glycine; 0.1% SDS; should be pH=8.8 without adjustment) at 20 V/cm for 30–45 min. After electrophoresis the gel was used for western blot (2.9.4) or stained with Coomassie staining solution (0.25% Coomassie Brilliant Blue R250 (Merck, Darmstadt, Germany); 45% ethanol; 10% Acetic Acid) for 30 min, followed by washing in destaining solution (15% ethanol; 15% Acetic acid) at room temperature until the protein bands were clear.

2.9.4 Western blot

Tank-blotting western transfer procedure was performed after SDS-PAGE (2.9.3). Eight pieces of whatman filter paper (GE Healthcare Life Sciences, Little Chalfont, United Kingdom) and a piece of PVDF membrane (GE Healthcare Life Sciences, Little Chalfont, United Kingdom) were cut to the same size as the gel. The dry PVDF membrane was activated in 100% methanol for 15 s and soaked with whatman paper in tank-blotting transfer buffer (25 mM Tris base; 150 mM glycine; 20% methanol; should be pH=8.3 without adjustment). Avoiding air bubbles, 4 sheets of filter paper were placed on the cathode (negative, usually black) and followed by the gel, the membrane, 4 sheets of filter paper were followed by, and finally the anode (positive, usually red). Proteins were transferred from the gel to the membrane in Rotiphorese PROclamp MINI Tank Blotting System (Roth, Karlsruhe, Germany) at 0.8 mA/cm^2 for 1 h. Anti-His HRP Conjugate blocking buffer (5 PRIME, Hamburg, Germany) was prepared during western transfer. 0.1 g Blocking Reagent was added into 20 mL 1 × Blocking Reagent buffer (5 PRIME, Hamburg, Germany) at 70 ℃ until dissolved [final concentration 5% (w/v)]. 200 µL 10% Tween-20 (final concentration 0.1%) was added and blocking buffer should be cooled to room temperature before use. After western transfer, the PVDF membrane was washed twice for 10 min each time with TBS buffer (10 mM Tris-Cl, pH=7.5; 150 mM NaCl) at room temperature and then incubated for 1 h in Anti-His HRP Conjugate blocking buffer at room temperature. The membrane was washed twice for 10 min each time in TBS-Tween/Triton buffer (20 mM Tris-Cl, pH=7.5; 500 mM NaCl; 0.05% Tween-20; 0.2% Triton X-100) at room temperature. The membrane was washed for 10 min with TBS buffer at room temperature. The membrane was incubated in Anti-His HRP Conjugate solution (1:1000–1:2000 dilution of conjugate stock solution in blocking buffer) at room temperature for 1 h or 4 ℃ overnight. The membrane was washed with TBS-Tween/Triton buffer and TBS buffer as mentioned above. Chemiluminescent detection was performed by SuperSignal West Pico Chemiluminescent Substrate kit (Thermo, Waltham, USA). Luminol/Enhancer Solution and Stable Peroxide Solution were mixed together at a 1:1 ratio and incubated with membrane for 5 min at room temperature. The membrane was covered by plastic wrap and exposed to X-ray film for 15 s – 1 min in the dark room.

2.10 Yeast-one-hybrid

The vector pHISi was used for reporter constructs of yeast one hybrid in this study. DNA fragments were inserted into multiple cloning sites (MCS) of pHISi by enzyme digestion and ligation cloning (2.8.1). The recombinant pHISi plasmids were linearized by *Xho*I restriction enzyme digestion (2.6.4) and integrate into the genome of yeast strain YM4271 (*his3* locus) (2.1.1 Table 1) (2.2.3). After transformation and integration, the yeast cells were selected on the SD/-Ura plates (2.1.2 Table 2). The positive colonies from SD/-Ura plates were picked into 1 mL sterile 1 × TE buffer (10 mM Tris-HCl; 1 mM EDTA; pH 7.5) or water and vortexed vigorously. 10 µL suspended yeast cells were spread on SD/–Ura or SD/–His selection plates (2.1.2 Table 2) and incubated at 30 ℃ for 2 −3 days.

2.11 Electrophoretic Mobility Shift Assay (EMSA)

Single-stranded DNA of *Ipt* promoter sequence was synthesized by Sigma (Sigma, St. Louis, USA). Two complementary oligonucleotides were mixed at a 1∶1 molar ratio in annealing buffer (10 mM Tris-Cl, pH=8.0; 1 mM EDTA; 50 mM NaCl). The mixture was incubated at 95 ℃ for 5 min and slowly cooled down to room temperature overnight. Double-stranded DNA was purified from agarose gel by QIAquick Gel Extraction Kit protocol (Qiagen, Hilden, Germany) (2.6.3) and labeled by T4 polynucleotide kinase (Fermentas, Thermo, Waltham, USA) with [gamma-^{32}P] adenosine 5'-triphosphate (ATP) (Hartmann Analytic GmbH, Braunschweig, Germany) as the probe. The labeled probe was purified by MicroSpin™ G-25 Columns (GE Healthcare Life Sciences, Little Chalfont, United Kingdom). 6% polyacrylamide native gel (50 mL per gel) was prepared as following at least 3 h before use.

10 × TBE (900 mM Tris-borate; 20 mM EDTA; pH 8.6)	2.5 mL
Rotiphorese Gel 40 (29∶1) (Roth, Karlsruhe, Germany)	7.5 mL
H_2O	40.0 mL
10% ammonium persulfate (APS)	500 µL
TEMED (Roth, Karlsruhe, Germany)	25 µL

About 5 ng labeled probe and 150–300 ng recombinant protein was mixed in the DNA-protein binding buffer (10 mM Tris-Cl pH8.0, 0.5 mM $ZnSO_4$, 0.25 mM DTT, 0.1 µg/µL poly [dI-dC], 5% glycerol). The binding reaction mixtures were incubated on ice for 30 min and loaded on 6% polyacrylamide gel and electrophoresed at 4 ℃ for 3 h at 20 V/cm in 0.5 × TBE buffer (45 mM Tris-borate and 1 mM EDTA; pH 8.6). After drying, gels were exposed for film autoradiography overnight.

2.12 Protoplast Transactivation (PTA) system

2.12.1 Luciferase reporter constructs

The background of luciferase reporter construct was pSAT (pSAT-2221) USER vector. DNA fragments of luciferase reporter gene and CAMV-terminator were amplified by Phusion Cx

polymerase using USER primers and subsequently inserted into pSAT by USER cloning to construct luc+/pSAT-2221 reporter plasmid (2.8.3). The sequence of forward USER primer was 5' GGCTTAAU-TAAGGATCCTTAATTAAACCTCAGC + luciferase reporter gene specific sequence 3'. The underlined sequence was used to generate a new PacI cassette at 5' upstream of luciferase reporter gene for next USER cloning. The promoters and 5' untranslated regions (5' UTR) of *IaaH*, *IaaM* and *Ipt* were inserted into the new Pac I cassette at 5' upstream of luciferase reporter gene by USER cloning for reporter construct of PTA system.

2.12.2 Transcription factor expression constructs

All the transcription factor expression constructs of PTA system were kindly provided by Prof. Wolfgang Dröge-Laser (Julius-von-Sachs-Institute, Pharmaceutical Biology, University of Wuerzburg, Wuerzburg, Germany) [156].

2.12.3 Protoplast isolation and transformation

Mesophyll protoplasts were isolated from rosette leaves of 4–5 weeks old *Arabidopsis* plants. The surfaces of leaves were cut in 1 mm distance with a razor blade and placed in a 55 mm diameter petri dish with 20 mL enzyme solution (1.5% cellulase R10; 0.4% macerozyme R10; 0.4 M mannitol, 20 mM KCl; 10 mM $CaCl_2$; 20 mM MES; 0.1% BSA). Vacuum infiltrate leaf strips for 15 min in the dark. Digestion was performed at room temperature overnight.

Released protoplasts were filtered through a 63 µm mesh into a 50 mL centrifuge tube, and dilute the enzyme-protoplast solution with an equal volume of W5 solution. The protoplasts were collected by centrifugation at 100 g for 2 min (↗0↘0). After discarding the supernatant the protoplasts were re-suspended in 10 mL W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES) and centrifuged at 100g for 1 min (↗0↘0). The protoplasts were re-suspended in 10 mL W5 solution and kept on ice for 5 h. For polyethylene glycol (PEG) transformation, protoplasts were re-suspended in MMG solution (0.4 M mannitol, 15 mM $MgCl_2$, 4 mM MES), at a concentration of 4×10^5/mL. Liquid handling was performed with a multichannel pipette and required special cell saver tips with wide orifices to avoid protoplast damaging. 2 µL plasmid DNA (1 µg per construct) and 30 µL protoplast suspension (1×10^4) were transferred to each well of a round bottom white standard microtiter plate (Nunc U96 MicroWell Polypropylene Plates, Thermo, Waltham, USA). Transformations were performed by adding 35 µL PEG solution (40% PEG 4000; 0.2 M mannitol; 0.1 M $CaCl_2$). Mixing was carried out by pipetting gently for 10 times. After incubation for 20 min, 120 µL W5 solution were added and mixed by pipetting gently for 5 times. Transfected protoplasts were collected by centrifugation of the microtiter plate at 100 g for 1 min (↗0↘0) and re-suspended in 100 µL WI solution (0.5 M mannitol; 20 mM KCl; 4 mM MES) containing appropriate phytohormone. Incubation carried out under controlled conditions in a growth cabinet or at room temperature overnight.

2.12.4 Luminescence Measurement

Dual luciferase measurements were performed using Renilla-Juice BIG KIT and Beetle-Juice BIG KIT (PJK GMBH, Kleinblittersdorf, Germany). The supernatant was removed after overnight incubation and 20 µL Lysis-Juice 2 (Renilla-Juice BIG KIT) was added to each well and mixed with protoplast by pipetting. The microtiter plate was centrifuged (4000 rpm × 10 min) after 15 min incubation on ice. 10 µL supernatant of each well was transferred to two new independent microtiter plates. 50 µL Renilla-Juice for renilla LUC and 50 µL Beetle-Juice for firefly LUC were added via the liquid handling robotic and the luminescence was measured by Robion Solaris plate reader luminometer (STRATEC Biomedical Systems AG, Birkenfeld, Germany). The relative activity was calculated as firefly-LUC/renilla-LUC values. The relative activity of firefly LUC reporter construct without any transcription factors expression constructs or treatments was set up to 1 fold. The fold increase in luminescence represented the relative activity induced by tested transcription factors or treatment. The means were calculated from more than three independent experiments.

2.13 Bimolecular Fluorescence Complementation (BiFC)

In order to construct cYFP or nYFP fusion proteins for BiFC experiments, which were driven by UBQ10 promoter, the full length of ARF or WRKY CDS was cloned into pSAT vector (Figure S4 pSAT #18 and Figure S5 pSAT #20) by USER cloning (2.8.3). Mesophyll protoplasts were used for BiFC assay (2.12.3). 20 µg plasmid DNA and 200 µL protoplast suspension (1×10^5 protoplasts) were transferred to a 13 mL tubes with round bottom (SARSTEDT, Nuembrecht, Germany). Transformations were performed by adding 220 µL PEG solution (40% PEG 4000; 0.2 M mannitol; 0.1 M $CaCl_2$). Mixing was carried out by gently tapping the tube 6–10 times. After incubation for 20 min, 880 µL W5 solution were added and mixed by gently tapping the tube 6–10 times. Transfected protoplasts were collected by centrifugation of the microtiter plate at 100 g for 1 min (↗0↘0) and re-suspended in 2 mL WI solution (0.5 M mannitol; 20 mM KCl; 4 mM MES) in each well of a 6-well tissue culture plate (Thermo, Waltham, USA). Incubation carried out under controlled conditions in a growth cabinet or at room temperature overnight. The protoplasts images were taken with a confocal laser scanning microscope (Leica, Wetzlar, Germany).

3. Results

3.1 Both *Agrobacterium tumefaciens* and *Arabidopsis thaliana* express *IaaH*, *IaaM* and *Ipt* transcripts

The expressions of the *IaaH, IaaM* and *Ipt* genes were determined in *Agrobacterium tumefaciens* by applying reverse transcription PCR (RT-PCR 2.6.6) and the transcript numbers in *Arabidopsis thaliana* were measured by quantitative real time PCR (qRT-PCR, 2.6.7), respectively. Total RNA was isolated from *A. tumefaciens* strain C58 treated with and without 100 µM acetosyrinzgone and cDNA was synthesized by using random hexamer primers (2.7.3, 2.7.4). PCR products of *IaaH* (597 bp), *IaaM* (309 bp) and *Ipt* (290 bp) were amplified from the cDNA of *A. tumefaciens* strain C58 (Figure 6A) with qRT-PCR primers listed in Table 5 (2.6.7.3). The same amount of total RNA, used for reverse transcription, was directly PCR amplified to prove that the total RNA did not contain DNA contaminations. The results showed that the transcripts of *IaaH, IaaM* and *Ipt* were detected in the cDNA of *A. tumefaciens* strain C58 (Figure 6A). In addition, there is no obvious difference in the samples treated with or without 100 µM acetosyringone which induces expression of virulence genes in *A. tumefaciens* (Figure 6A). Quantification of the transcripts of the three oncogenes in 25-day-old *Arabidopsis* crown gall tumor tissues was performed with tumor cDNA (2.7.2) and the same primers as used for *A. tumefaciens* cDNA (2.6.7). The relative transcript numbers of the three oncogenes revealed that transcription of the *IaaH* and *IaaM* genes were much lower than that of *Ipt* (Figure 6B).

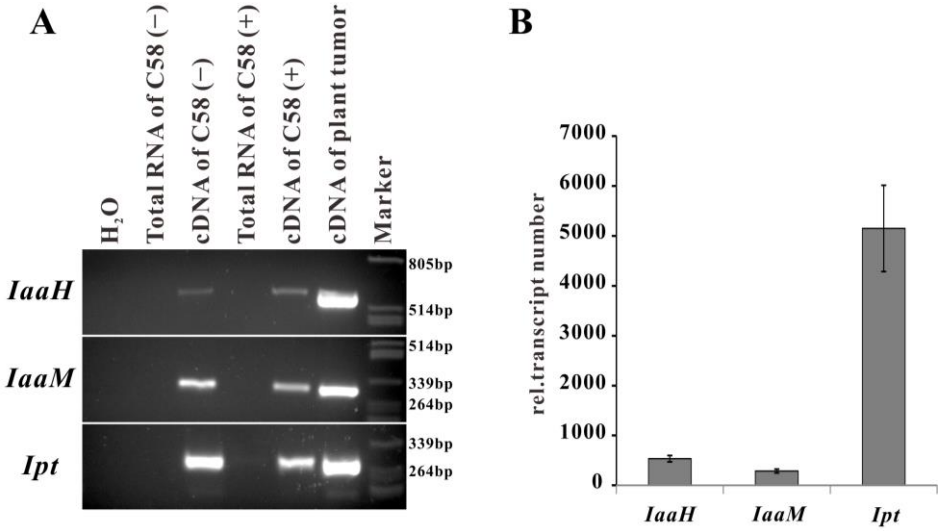


Figure 6. Oncogenes are expressed in the *A. tumefaciens* strain C58 and *Arabidopsis* crown gall tumors.
(A). *IaaH, IaaM* and *Ipt* were amplified from cDNA of strain C58 treated with (+) or without (−) acetosyringone. Water (H_2O) and total RNA served as negative controls for DNA contaminations. cDNA of *Arabidopsis* crown galls was used as a positive control. The fragment sizes of the marker bands are indicated at the right site. (B) Relative numbers of *IaaH, IaaM* and *Ipt* transcripts in crown gall tumors 25 days after inoculation of *A. tumefaciens* strain C58 into *Arabidopsis* inflorescence stems. Relative transcript numbers were quantified by qRT-PCR and normalized to 10,000 molecules of *ACTIN2/8*. Bars show mean values (±SD) of three independent samples.

3.2 Intergenic regions between oncogenes function as promoters in plant cells

The CDS of the three oncogenes *IaaH*, *IaaM* and *Ipt* from the nopaline Ti plasmid pTiC58, are arranged in the T-DNA region one after the other and interrupted by two non-coding intergenic regions (IGR1 and IGR2; Figure 7A). *IaaM* and *Ipt* genes are transcribed from the sense strand, whereas *IaaH* gene is encoded on the antisense strand. IGR1 comes along in two directions: one is 5' upstream of the transctiption start site (TTS) of the *IaaH* CDS (IGR1a) and the other 5' of *IaaM* (IGR1b). In order to prove, if the IGRs function as promoters in plant cells, the full lengths of IGR1a, IGR1b and IRG2 were fused with the CDS of the green fluorescent protein (GFP) and transformed into plant cells by inoculating *Arabidopsis* root segments with the virulent *A. tumefaciens* strain C58. Strain C58 then harbored the binary vector with the IGR-GFP construct and the its virulent Ti-plasmid. Root segments transformed with a 2 × 35S CaMV promoter::GFP construct was used as positive control and the GFP-CDS without promoter served as negative control. Stable transformed *Arabidopsis* crown gall tumor cell lines, which arose from the infection of the root segments with the transgenic and virulent *A. tumefaciens* strains, were analyzed in this study. Detection of GFP fluorescence in the *IGR1a::GFP*, *IGR1b::GFP* and *IGR2::GFP* cell lines demonstrated that the IGRs functioned as promoters in plant cells and that IGR1 is a bidirectional promoter which can drive transcription of *IaaH* and *IaaM* (Figure 7B).

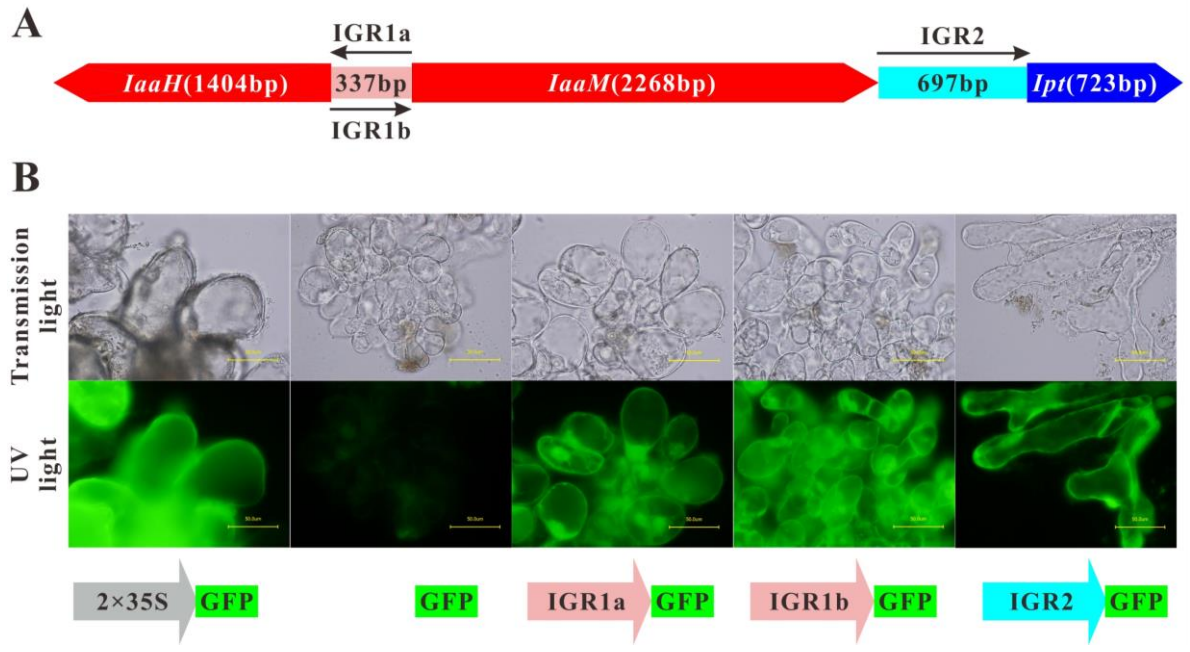


Figure 7. IGR1 and IGR2 function as promoters in *Arabidopsis* cells.
(A) Arrangement of the coding sequences (CDS) of the *IaaH*, *IaaM* and *Ipt* oncogenes separated by intergenic regions (IGRs) in the T-DNA region of the Ti-plasmid of *Agrobacterium tumefaciens* strain C58, pTiC58. (B) Green fluorescence indicates that IGR1 and IGR2 drive GFP expression in plant cells. For IGR1 upstream of *IaaH* (IGR1a) and *IaaM* (IGR1b) both directions were analyzed. 2 × 35s promoter was used as positive control and the GFP-CDS without promoter served as negative control. Top row: images from crown gall tumor callus cells were taken in the transmission microscopy mode and bottom row, in the UV light mode. Bars = 50 µm. The tumor cell culture lines were generated by Chil-Woo Lee (Julius-von-Sachs-Institute, Department of Molecular

Plant Physiology and Biophysics, University of Wuerzburg, Germany, current address: Leibniz Institute of Plant Biochemistry, Halle /Saale, Germany)

Since IGR1a, IGRb and IGR2 function as promoters, their sequences should contain the core promoter elements. Therefore, the transcription start sites (TSSs) of the *IaaH*, *IaaM* and *Ipt* genes were determined using the 5' RACE assay (2.7.5). Table 6 lists the consensus sequences around the TSSs, which are the typical eukaryotic initiator (Inr) boxes (YYANWYY, TSS is underlined, Y = C/T, W = A/T, N = A/G/C/T) and in agreement with the plant specific "YR Rule" (YR, TSS is underlined, Y = C/T, R = A/G) [56,57]. The translational start code of the *IaaH*, *IaaM* and *Ipt* CDSs are localized at +12 bp, +26 bp and +44 bp, respectively. The TATA boxes, which are binding sites for the general transcription factors, were found in the promoter regions between −25 bp to −35 bp of the three promoters. In addition, CAAT boxes, which are present in many eukaryotic promoters, are localized at about −70 bp upstream of the TSSs within the oncogene promoter regions (Table 6).

Table 6 *Cis*-regulatory sequence elements within the oncogene promoters

Regulatory sequences	*IaaH*	*IaaM*	*Ipt*
Inr box (YY**A**NWYY)	−2 CC**A**AACC +5	−2 CT**A**CACA +5	−2 CT**A**ATCC +5
Start coden ATG	+12 ATG	+26 ATG	+44 ATG
TATA box (TATAAA)	−36 TATATT −31 [1]	−32 TAAATA −27 [2]	−29 TATAAC −24 [3456]
CAAT box (GGNCAATCN)	−66 CCAAT −62 [1]	−75 CCATT −71 [2]	−72 GGTAAAGCC −64 [3] −49 AAGGAATCT −41 [4, 5]

Positive numbers indicate the positions downstream and negative numbers the positions upstream of the TSSs (+1). Y = C/T, W = A/T, N = A/G/C/T. 1[165].; 2. [166]; 3. [167]; 4. [168]; 5. [169]; 6. [170]

3.3 Core promoter elements are conserved in the *IaaH*, *IaaM* and *Ipt* promoters

An alignment of the oncogene promoters and 5' untranslated region (5' UTR) of the homologous sequences of the T-DNA region from two nopaline-type (pTiC58, pTiSAKURA), three octopine-type (pTiA6NC, pTiAch5, pTi15955) and one agropine-type Ti plasmids (pTiBo542), shows that the TTSs (arrows), TATA boxes and CAAT boxes of *IaaH* and *IaaM* promoters are conserved (Figure 8, 9). Within the *Ipt* promoter sequence of the T-DNA region of the three Ti plasmids the positions of the TSS and TATA box are also conserved (Figure 10). The first predicted CAAT box (GGTAAAGCC, −72 to −64 bp) is conserved in the octopine and nopaline Ti plasmids, but was not found in the agrocinopine-type *Ipt* promoter; the second predicted CAAT box (AAGGAATCT, −49 to −41 bp) is only conserved in the *Ipt* promoter of the nopaline-type Ti-plasmid (Figure 10). These *cis*-regulatory sequence elements contribute to the expression of the three oncogenes in plant cells. In addition, the alignment of the *Ipt* promoters revealed that a 60 bp sequence between −193 bp and −134 bp from the

translational start codon ATG of *Ipt* oncogene in pTiC58, which is −150 bp to −91 bp from the TTS in IGR2, is conserved and named as *Ipt* element in this study (Figure 10).

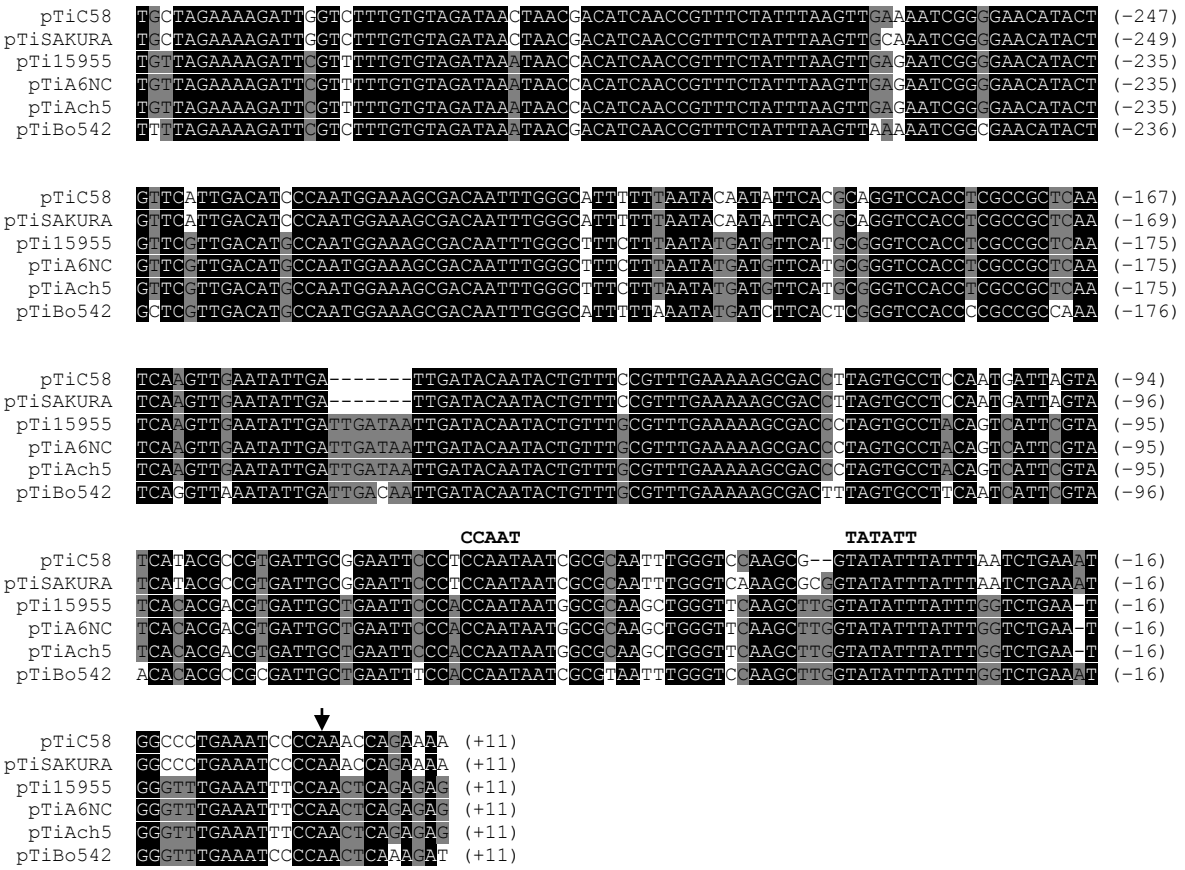


Figure 8. Alignment of *IaaH* promoter sequences and 5'untranslated region (5' UTR) from different *A. tumefaciens* strains.
T-DNAs of the nopaline-type (pTiC58, pTiSAKURA), the octopine-type (pTi15955, pTiA6NC, pTiAch5) and one of the agropine-type Ti plasmids (pTiBo542). Arrows indicate the position of the transcription start sites (TSS). Numbers at the right site indicate the nucleotide positions from the TSS. TATA box and CAAT box sequences are written above the aligned sequences.

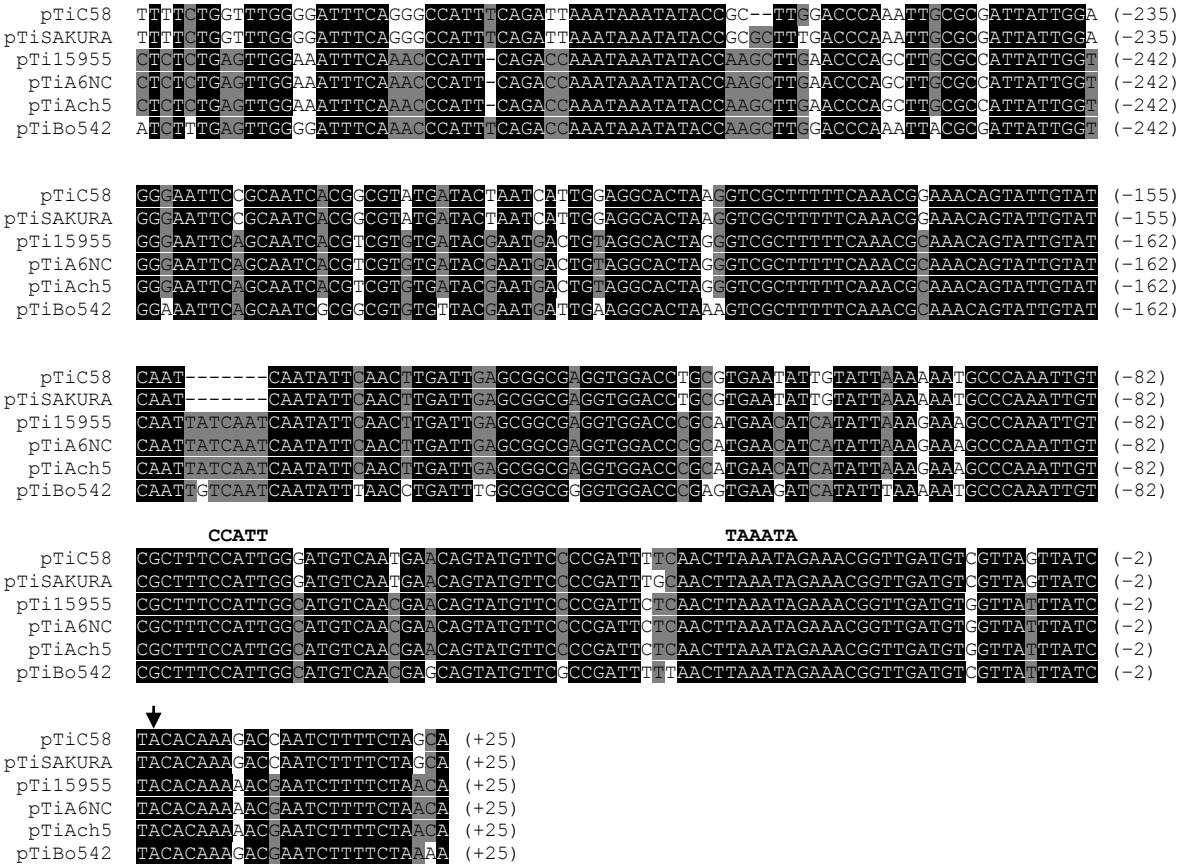
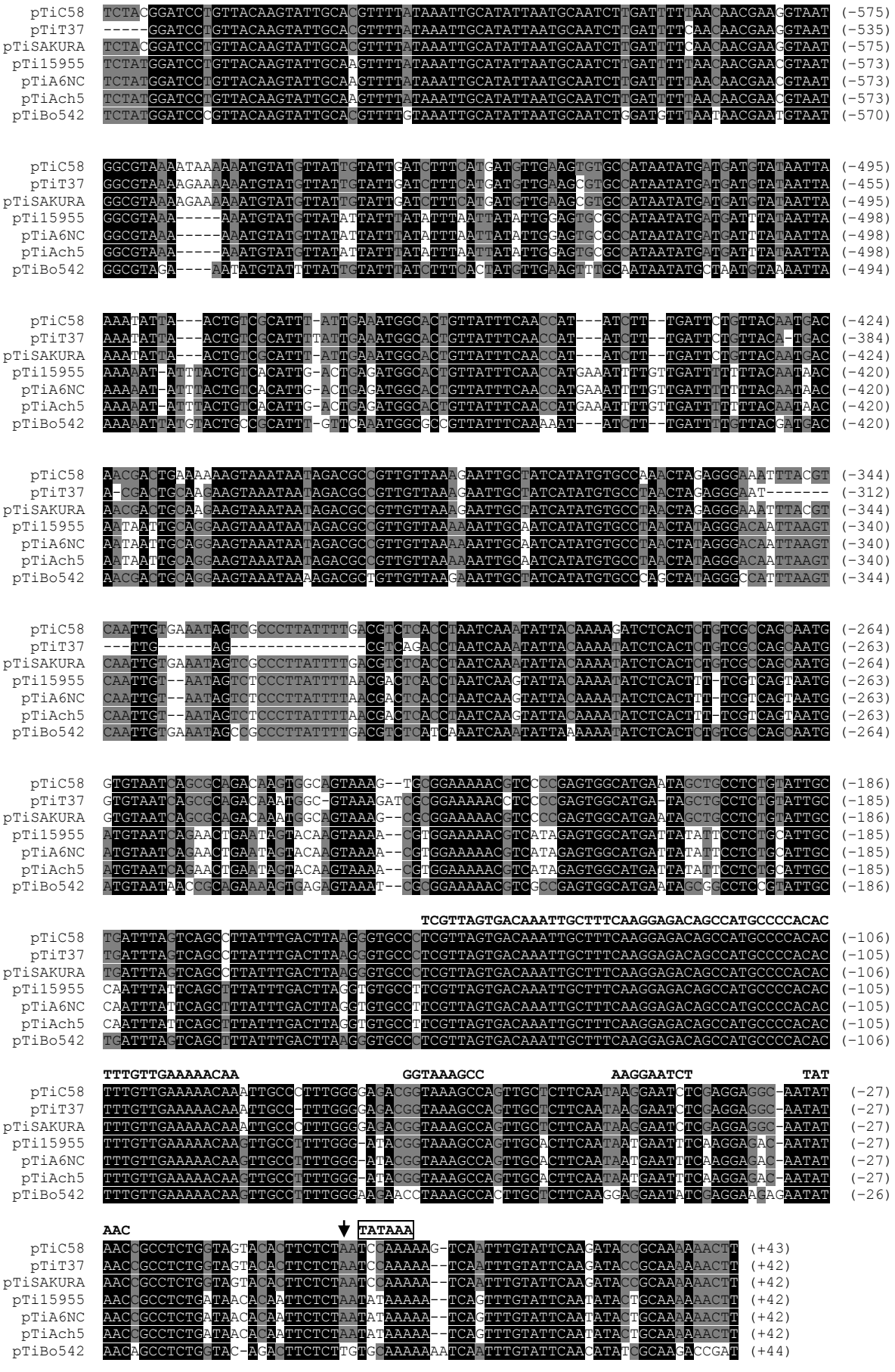


Figure 9. Alignment of *IaaM* promoter sequences and 5' untranslated region (5' UTR) from different *A. tumefaciens* strains.
T-DNAs of the nopaline-type (pTiC58, pTiSAKURA), the octopine-type (pTi15955, pTiA6NC, pTiAch5) and one of the agropine-type Ti plasmids (pTiBo542). Arrows indicate the position of the transcription start sites (TSS). Numbers at the right site indicate the nucleotide positions from the TSS. TATA box and CAAT box sequences are written above the aligned sequences.


pTiC58 TCTACGGATCCTGTTACAAGTATTGCACGTTTTATAAATTGCATATTAATGCAATCTTGATTTTAACAACGAAGGTAAT (-575)
pTiT37 -----GGATCCTGTTACAAGTATTGCACGTTTTATAAATTGCATATTAATGCAATCTTGATTTTCAACAACGAAGGTAAT (-535)
pTiSAKURA TCTACGGATCCTGTTACAAGTATTGCACGTTTTATAAATTGCATATTAATGCAATCTTGATTTTCAACAACGAAGGTAAT (-575)
pTi15955 TCTATGGATCCTGTTACAAGTATTGCAAGTTTTATAAATTGCATATTAATGCAATCTTGATTTTTAACAACGAACGTAAT (-573)
pTiA6NC TCTATGGATCCTGTTACAAGTATTGCAAGTTTTATAAATTGCATATTAATGCAATCTTGATTTTTAACAACGAACGTAAT (-573)
pTiAch5 TCTATGGATCCTGTTACAAGTATTGCAAGTTTTATAAATTGCATATTAATGCAATCTTGATTTTTAACAACGAACGTAAT (-573)
pTiBo542 TCTATGGATCCGGTTACAAGTATTGCACGTTTTGTAAATTGCATATTAATGCAATCTGGATGTTTAATAACGAATGTAAT (-570)

pTiC58 GGCGTAAAATAAAAAATGTATGTTATTGTATTCATCTTTCATGATGTTGAAGTGTGCCATAATATGATGATGTATAATTA (-495)
pTiT37 GGCGTAAAAGAAAAAATGTATGTTATTGTATTGATCTTTCATGATGTTGAAGCGTGCCATAATATGATGATGTATAATTA (-455)
pTiSAKURA GGCGTAAAAGAAAAAATGTATGTTATTGTATTGATCTTTCATGATGTTGAAGCGTGCCATAATATGATGATGTATAATTA (-495)
pTi15955 GGCGTAAA-----AAATGTATGTTATATTATTTATATTTAATTATATTGGAGTGCGCCATAATATGATGATTTATAATTA (-498)
pTiA6NC GGCGTAAA-----AAATGTATGTTATATTATTTATATTTAATTATATTGGAGTGCGCCATAATATGATGATTTATAATTA (-498)
pTiAch5 GGCGTAAA-----AAATGTATGTTATATTATTTATATTTAATTATATTGGAGTGCGCCATAATATGATGATTTATAATTA (-498)
pTiBo542 GGCGTAGA----AATATGTATTTTATTGTATTTATCTTTCACTATGTTGAAGTTTGCAATAATATGCTAATGTAAAATTA (-494)

pTiC58 AAATATTA---ACTGTCCCATTT-ATTGAAATGGCACTGTTATTTCAACCAT---ATCTT--TGATTCTGTTACAATGAC (-424)
pTiT37 AAATATTA---ACTGTCCCATTTTATTGAAATGGCACTGTTATTTCAACCAT---ATCTT--TGATTCTGTTACA-TGAC (-384)
pTiSAKURA AAATATTA---ACTGTCCCATTT-ATTGAAATGGCACTGTTATTTCAACCAT---ATCTT--TGATTCTGTTACAATGAC (-424)
pTi15955 AAAAAT-ATTTACTGTCACATTG-ACTGAGATGGCACTGTTATTTCAACCATGAAATTTTGTTGATTTTTTTACAATAAC (-420)
pTiA6NC AAAAAT-ATTTACTGTCACATTG-ACTGAGATGGCACTGTTATTTCAACCATGAAATTTTGTTGATTTTTTTACAATAAC (-420)
pTiAch5 AAAAAT-ATTTACTGTCACATTG-ACTGAGATGGCACTGTTATTTCAACCATGAAATTTTGTTGATTTTTTTACAATAAC (-420)
pTiBo542 AAAAATTATGTACTGCCCCATTT-GTTCAAATGGCGCCGTTATTTCAAAAT---ATCTT--TGATTTTGTTACGATGAC (-420)

pTiC58 AACGACTGAAAAAAGTAAATAATAGACGCCGTTGTTAAAGAATTGCTATCATATGTGCCAAACTAGAGGGAAATTTACGT (-344)
pTiT37 A-CGACTGCAACAAGTAAATAATAGACGCCGTTGTTAAAGAATTGCTATCATATGTGCCTAACTAGAGGGAAT------- (-312)
pTiSAKURA AACGACTGCAACAAGTAAATAATAGACGCCGTTGTTAAAGAATTGCTATCATATGTGCCTAACTAGAGGGAAATTTACGT (-344)
pTi15955 AATAATTGCAGGAAGTAAATAATAGACGCCGTTGTTAAAAAATTGCAATCATATGTGCCTAACTATAGGGACAATTAAGT (-340)
pTiA6NC AATAATTGCAGGAAGTAAATAATAGACGCCGTTGTTAAAAAATTGCAATCATATGTGCCTAACTATAGGGACAATTAAGT (-340)
pTiAch5 AATAATTGCAGGAAGTAAATAATAGACGCCGTTGTTAAAAAATTGCAATCATATGTGCCTAACTATAGGGACAATTAAGT (-340)
pTiBo542 AACGACTGCAGGAAGTAAATAAAGACGCTGTTGTTAAGAAATTGCTATCATATGTGCCCAGCTATAGGGCCATTTAAGT (-344)

pTiC58 CAATTGTGAAATAGTCGCCCTTATTTTGACGTCTCACCTAATCAAATATTACAAAAGATCTCACTCTGTCGCCAGCAATG (-264)
pTiT37 ---TTG------AG--------------CGTCAGACCTAATCAAATATTACAAAATATCTCACTCTGTCGCCAGCAATG (-263)
pTiSAKURA CAATTGTGAAATAGTCGCCCTTATTTTGACGTCTCACCTAATCAAATATTACAAAATATCTCACTCTGTCGCCAGCAATG (-264)
pTi15955 CAATTGT--AATAGTCTCCCTTATTTTAACGACTCACCTAATCAAGTATTACAAAATATCTCACTTT-TCGTCAGTAATG (-263)
pTiA6NC CAATTGT--AATAGTCTCCCTTATTTTAACGACTCACCTAATCAAGTATTACAAAATATCTCACTTT-TCGTCAGTAATG (-263)
pTiAch5 CAATTGT--AATAGTCTCCCTTATTTTAACGACTCACCTAATCAAGTATTACAAAATATCTCACTTT-TCGTCAGTAATG (-263)
pTiBo542 CAATTGTGAAATAGCCGCCCTTATTTTGACGTCTCATCAAATCAAATATTAAAAATATCTCACTCTGTCGCCAGCAATG (-264)

pTiC58 GTGTAATCAGCGCAGACAAGTGGCAGTAAAG--TGCGGAAAAACGTCCCGAGTGGCATGAATAGCTGCCTCTGTATTGC (-186)
pTiT37 GTGTAATCAGCGCAGACAAATGGC-GTAAAGATCGCGGAAAAACCTCCCCGAGTGGCATGA-TAGCTGCCTCTGTATTGC (-185)
pTiSAKURA GTGTAATCAGCGCAGACAAATGGCAGTAAAG--CGCGGAAAAACGTCCCGAGTGGCATGAATAGCTGCCTCTGTATTGC (-186)
pTi15955 ATGTAATCAGAACTGAATAGTACAAGTAAAA--CGTGGAAAAACGTCATAGAGTGGCATGATTATATTCCTCTGCATTGC (-185)
pTiA6NC ATGTAATCAGAACTGAATAGTACAAGTAAAA--CGTGGAAAAACGTCATAGAGTGGCATGATTATATTCCTCTGCATTGC (-185)
pTiAch5 ATGTAATCAGAACTGAATAGTACAAGTAAAA--CGTGGAAAAACGTCATAGAGTGGCATGATTATATTCCTCTGCATTGC (-185)
pTiBo542 ATGTAATAACCGCAGAAAAGTGAGAGTAAAT--CGCGGAAAAACGTGGCCGAGTGGCATGAATAGCGCCTCCGTATTGC (-186)

TCGTTAGTGACAAATTGCTTTCAAGGAGACAGCCATGCCCCACAC
pTiC58 TGATTTAGTCAGCCTTATTTGACTTAAGGGTGCCCTCGTTAGTGACAAATTGCTTTCAAGGAGACAGCCATGCCCCACAC (-106)
pTiT37 TGATTTAGTCAGCCTTATTTGACTTAAGGGTGCCCTCGTTAGTGACAAATTGCTTTCAAGGAGACAGCCATGCCCCACAC (-105)
pTiSAKURA TGATTTAGTCAGCCTTATTTGACTTAAGGGTGCCCTCGTTAGTGACAAATTGCTTTCAAGGAGACAGCCATGCCCCACAC (-106)
pTi15955 CAATTTATTCAGCTTTATTTGACTTAGGTGTGCCTTCGTTAGTGACAAATTGCTTTCAAGGAGACAGCCATGCCCCACAC (-105)
pTiA6NC CAATTTATTCAGCTTTATTTGACTTAGGTGTGCCTTCGTTAGTGACAAATTGCTTTCAAGGAGACAGCCATGCCCCACAC (-105)
pTiAch5 CAATTTATTCAGCTTTATTTGACTTAGGTGTGCCTTCGTTAGTGACAAATTGCTTTCAAGGAGACAGCCATGCCCCACAC (-105)
pTiBo542 TGATTTAGTCAGCTTTATTTGACTTAAGGGTGCCCTCGTTAGTGACAAATTGCTTTCAAGGAGACAGCCATGCCCCACAC (-106)

TTTGTTGAAAAACAA GGTAAAGCC AAGGAATCT TAT
pTiC58 TTTGTTGAAAAACAAATTGCCCTTTGGGGAGACGGTAAAGCCAGTTGCTCTTCAATAAGGAATCTCGAGGAGGC-AATAT (-27)
pTiT37 TTTGTTGAAAAACAAATTGCC-TTTGGGGAGACGGTAAAGCCAGTTGCTCTTCAATAAGGAATCTCGAGGAGGC-AATAT (-27)
pTiSAKURA TTTGTTGAAAAACAAATTGCCCTTTGGGGAGACGGTAAAGCCAGTTGCTCTTCAATAAGGAATCTCGAGGAGGC-AATAT (-27)
pTi15955 TTTGTTGAAAAACAAGTTGCCTTTGGG-ATACGGTAAAGCCAGTTGCACTTCAATAATGAATTTCAAGGAGAC-AATAT (-27)
pTiA6NC TTTGTTGAAAAACAAGTTGCCTTTGGG-ATACGGTAAAGCCAGTTGCACTTCAATAATGAATTTCAAGGAGAC-AATAT (-27)
pTiAch5 TTTGTTGAAAAACAAGTTGCCTTTGGG-ATACGGTAAAGCCAGTTGCACTTCAATAATGAATTTCAAGGAGAC-AATAT (-27)
pTiBo542 TTTGTTGAAAAACAAGTTGCCTTTGGGAAGAACCTAAAGCCACTTGCTCTTCAAGGAGGAATATCGAGGAAGAGAATAT (-26)

AAC TATAAA
pTiC58 AACGCCTCTGGTAGTACACTTCTCTAATCCAAAAAG-TCAATTTGTATTCAAGATACCGCAAAAAACTT (+43)
pTiT37 AACGCCTCTGGTAGTACACTTCTCTAATCCAAAAA--TCAATTTGTATTCAAGATACCGCAAAAAACTT (+42)
pTiSAKURA AACGCCTCTGGTAGTACACTTCTCTAATCCAAAAA--TCAATTTGTATTCAAGATACCGCAAAAAACTT (+42)
pTi15955 AACGCCTCTGATAACACAATTCTCTAATATAAAAA--TCAGTTTGTATTCAATATACTGCAAAAAACTT (+42)
pTiA6NC AACGCCTCTGATAACACAATTCTCTAATATAAAAA--TCAGTTTGTATTCAATATACTGCAAAAAACTT (+42)
pTiAch5 AACGCCTCTGATAACACAATTCTCTAATATAAAAA--TCAGTTTGTATTCAATATACTGCAAAAAACTT (+42)
pTiBo542 AACAGCCTCTGGTAC-AGACTTCTCTTGTGCAAAAAAATCAATTTGTATTCAACATATCGCAAAGACCGAT (+44)

Figure 10. Alignment of *Ipt* promoter sequences and 5' untranslated region (5' UTR) from different *A. tumefaciens* strains
T-DNAs of the nopaline-type (pTiC58, pTiT37, pTiSAKURA), the octopine-type (pTi15955, pTiA6NC, pTiAch5) and one of the agropine-type Ti plasmids (pTiBo542). Arrows indicate the position of the transcription start sites (TSS). Numbers at the right site indicate the nucleotide positions from the TSS. TATA box and CAAT box sequences are written above the aligned sequences. The boxed "TATAAA" sequence indicates the TATA box which is only conserved in octopine Ti plasmids. The sequence from −150 to −91 shows the conserved region, which is named as *Ipt* element.

3.4 Transcription factors potentially involved in regulation of oncogene expression

Cis-regulatory sequence binding elements for transcription factors in the *IaaH*, *IaaM* and *Ipt* promoters were determined in forward and reverse direction using PLACE (http://www.dna.affrc.go.jp/PLACE/index.html) [171-173]. Several binding elements for different transcription factor families including MYB, DOF, WRKY, bHLH, ARR1 and ARF were localized within the *Ipt* promoter (Table 7). In the *IaaH* and *IaaM* promoters eight binding elements for ARR1 were enriched. The cis-regulatory sequence elements might function as binding sites for transcription factors to enhance transcription of the three oncogenes.

Table 7 Cis-regulatory DNA elements for binding of transcription factors in the upstream regions of TSSs of the oncogenes.

Transcription factor family		*IaaH* promoter (−1 to −301)	*IaaM* promoter (−1 to −301)	*Ipt* promoter (−1 to −654)	*Ipt* promoter reverse (−1 to −654)
MYB	MYBCORE (CNGTTR or AACGG)	0	2	5	3
	MYB2AT (YAACKG)	0	0	1	2
DOF (AAAG)		2	0	5	5
WRKY (TGAC)		1	0	4	2
bHLH (CANNTG)		0	0	4	4
ARR1 (GATT)		3	5	3	4
ARF (TGTCNC or TGTCTN)		0	1	2	3

Y = C/T, K = G/T, W = A/T, R = A/G, N = A/G/C/T

In order to identify potential transcription factors, which may be involved in activation of oncogene expression, microarray data of *Arabidopsis* crown galls [28,174] were analyzed based on the *Arabidopsis thaliana* transcription factors listed in the Plant Transcription Factor Database v3.0 [58] (http://planttfdb.cbi.pku.edu.cn/index.php?sp=Ath). A total of 151 transcription factor genes were found to be differentially transcribed between inflorescence stem tissue inoculated with the virulent *A. tumefaciens* strain C58 and non-inoculated stem tissue (6.1 Table S1; fold change ≥ 2 or ≤ 0.5, p value < 0.01). Three genes were up-regulated 3 hours postinoculation (hpi), including WRKY53 (2.47 folds change), WRKY40 (2.22 folds change), and NAC102 (2.18 folds change). WRKY53 was also up-regulated 3 hpi by the disarmed *A. tumefaciens* strain GV3101 (2.37 folds change). Six days

postinoculation (dpi), six transcription factors were up-regulated and six down-regulated (6.1 Table S1). In 35-day-old *Arabidopsis* crown gall material of strain C58 141 transcription factor genes were transcriptionally changed compared to the reference material 35 days post wounding (dpw). Among of them, 74 transcription factors were up-regulated and 67 down-regulated (6.1 Table S1). These transcription factor genes belong to various families, such as WRKY, MYB, DOF, and NAC (6.1 Table S1). Considering both, (*i*) the DNA binding elements and (*ii*) the microarray data, MYB, DOF, WRKY, bHLH, ARR1 and ARF families are likely the potential candidates being involved in regulation of *Ipt* expression, whereas ARR1 might regulate transcription of the *IaaH* or *IaaM* genes.

3.5 Identification of the transcription factors that regulate oncogene expression

3.5.1 Application of the yeast-one-hybrid assay failed to identify transcription factors that regulate the oncogene promoters

The most common method for screening and identification of transcription factors is the Yeast-One-Hybrid (Y-1-H) assay. A library for the Y-1-H assay, containing approximately 1500 transcription factor cDNAs of *Arabidopsis thaliana*, was provided by N. Mitsuda (Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan) [155]. The full lengths of *IaaH* (337 bp), *IaaM* (337bp) and *Ipt* (697 bp) promoters and 5' UTR of pTiC58 oncogenes were inserted into the pHISi vector 5' upstream of a minimal promoter (Pmini, Figure 11A). The pHISi was used as promoterless construct. The promoterless and recombinant vectors were transformed and integrated into the genome of the yeast strain YM4271 (2.10). Both yeast reporter genes, *URA3* (Uracil) and *HIS3* (Histidine) in pHISi are used as selection markers for successful promoter sequence integration. Without activation by binding of a transcription factor to the inserted promoters, Pmini containing a TATA box and TSS drives constitutive but low HIS3 expression. Usually this low expression is strong enough for growth of yeast cells on a SD/-His (Synthetic Defined medium without histidine) selection plate. The low background expression of *HIS3* can be completely inhibited by adding 3-amino-1,2,4-triazole (3-AT) to the medium, a competitive inhibitor of the *HIS3* gene product [175]. Growth of all recombinant yeast strains on plates containing SD/-Ura selection medium indicated that the vectors had been transformed and integrated successfully, which is due to the URA reporter gene in the vector (Figure 11B). Unfortunately, the *HIS3* reporter gene was not expressed in yeast cells, when the recombinant vectors, harboring the oncogene promoters *IaaH*, *IaaM*, and *Ipt* were transformed. The yeast strains did not grow on the SD/-His selection plate even when the 3-AT inhibitor was lacking in the medium (Figure 11B). This result indicates that expression of the *HIS3* reporter gene is inhibited.

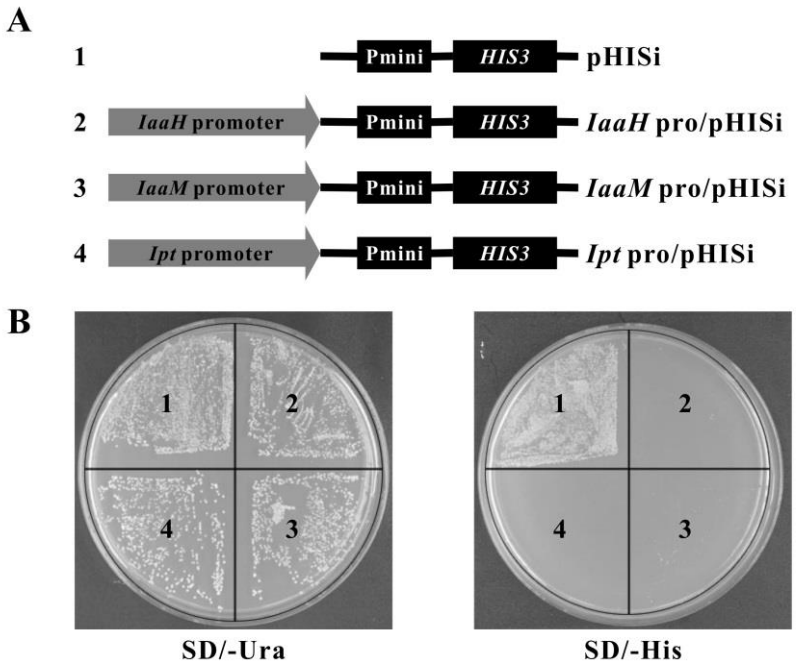


Figure 11. Yeast-one-hybrid assay with oncogene promoters.
(A) Schematic representation of the pHISi vector constructs, consisting of the yeast *HIS3* reporter gene, minimal promoter (Pmini) and no promoter (1) or the full length oncogene promoters (2–3). (B) Growth of the yeast strains containing the constructs 1–4 on SD/-Ura (Synthetic Defined medium without Uracil) or SD/-His (Synthetic Defined medium without histidine) selection plates lacking the inhibitor 3-amino-1,2,4-triazole (3-AT).

The three oncogenes promoters contain TATA boxes and TSSs (Table 6). In order to prove, whether two TATA boxes and the TSSs are a problem for expression of the *HIS3* reporter gene in yeast cells, Pmini was deleted (Figure 12A). These strains still did not grow on the SD/-His selection plate without addition of the 3-AT inhibitor (Figure 12B). Next, the TATA box and TSS of the *Ipt* promoter were separated at the *Xho*I site (−42 bp to −37 bp from TSS) from the oncogene promoter sequences and the remaining part (−654 bp to −37 bp) was inserted upstream of Pmini. The strain, containing the ΔTATA *Ipt* promoter, also did not grow on the SD/-His selection plate without addition of the 3-AT inhibitor (Figure 12B). These results suggest that inhibition of the *HIS3* reporter gene expression in yeast is not due to the two sets of TATA boxes and TSSs.

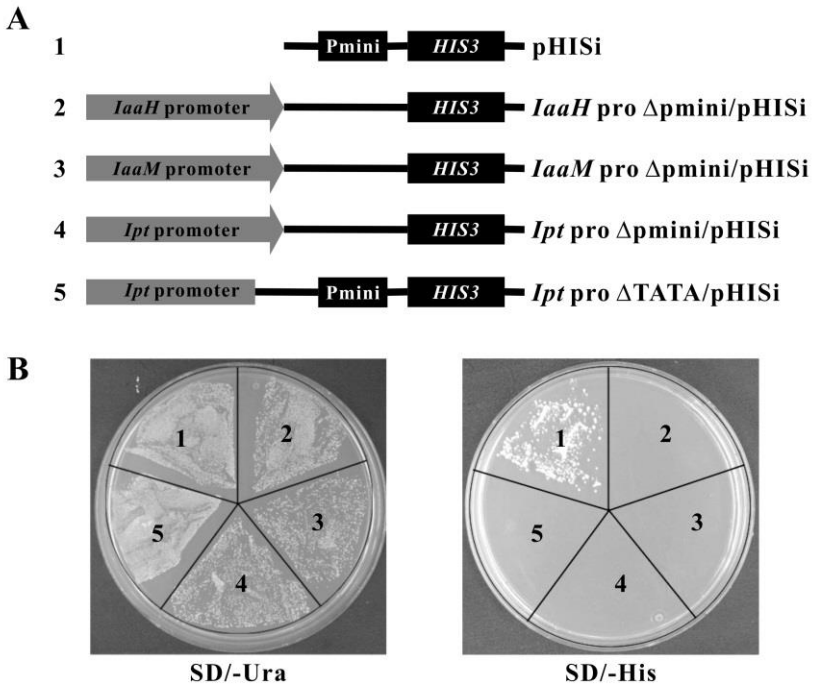


Figure 12. Yeast one-hybrid assay with deletions of the Pmini and the Ipt promoter.
(A) Schematic representation of the pHISi vector constructs without or with deletions of Pmini (ΔPmini, 1–4) or the TATA box (ΔTATA) and transcription start site (TSS) of the Ipt promoter (5), inserted upstream of the yeast *HIS3* reporter gene. (B) Growth of the yeast strains on SD/-Ura (Synthetic Defined medium without Uracil) or SD/-His (Synthetic Defined medium without histidine) selection plates lacking the inhibitor 3-amino-1,2,4-triazole (3-AT).

Finally different truncations of the oncogene promoters were tested for their activity in the Y-1-H assay. The IGR1 was cut with *EcoRI*, a restriction site, which is located −76 bp to −71 bp from the TSS of *IaaH* and −232 bp to −227 bp from that of the *IaaM* gene. The yeast strains, which harbored the promoter fragment, *IaaH* pro ΔEcoRI/pHISi, expressed the *HIS3* reporter gene and grew on SD/-His selection plate, but not those which contain the *IaaM* pro ΔEcoRI/pHISi (Figure 13A, B). The conserved region in the *Ipt* promoter (named as *Ipt* element, Figure 10) was used to generate the *Ipt* element/pHISi reporter plasmid. This *Ipt* promoter element drove *HIS3* reporter gene expression (Figure 13B). The strains with the full-length promoters of the oncogenes upstream of the HIS3 reporter sequence did again not grow on SD/-His selection medium. These results imply that unknown repressor in the yeast strain YM4271 used in these studies seems to bind the three promoters and inhibit expression of the *HIS3* reporter gene.

It has been reported that the Multicopy Inhibitor of Galactose (MIG1) protein from yeast *Saccharomyces cerevisiae* could bind the *cyt-1* element [154]. The sequence of *cyt-1* element is 5'-TGCCCCACACTTTGTTG-3', and it is located in the conserved region of the *Ipt* promoter (from −99 bp to −115 bp in our study). An as yet unknown protein from tobacco nuclear extracts, designated as CBF (*cyt-1* binding factor), can bind the cyt-1 element [153]. MIG1 is a C_2H_2 type zinc finger protein and binds to a GCGGGG motif [176]. MIG1 functions as a transcriptional repressor in yeast [177,178].

However, the Y-1-H assay shows that the constructs of the *Ipt* element in pHISi plasmid could drive *HIS3* reporter gene expression in yeast YM4271 (Figure 13), so MIG1 is not the reason why expression of the *HIS3* reporter is repressed. Schouten and coworkers also proved some yeast activators bound *cyt-1* element [154]. In summary, some proteins in yeast can bind, and even activate or repress *cyt-1* element, so searching for the binding activity of additional plant transcription factor for *Ipt* promoter is not possible [179]. The failure of *IaaH* and *IaaM* promoter in Y-1-H may be due to the same reason.

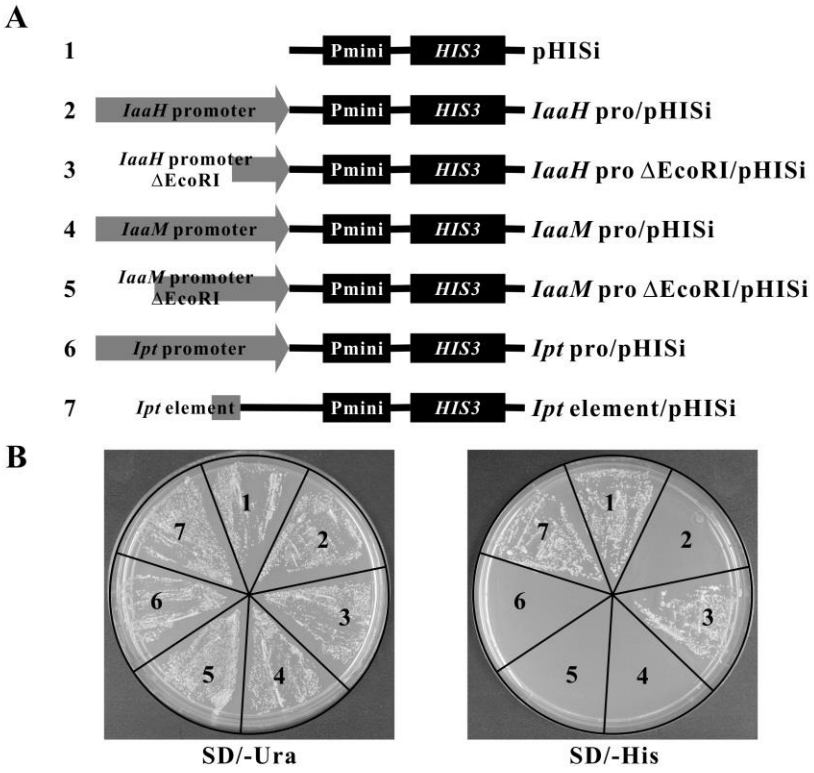


Figure 13. Yeast-one-Hybrid assay with truncations of the oncogene promoters.
(A) Schematic representation of the pHISi vector, containing the minimal promoter (Pmini, 1), full length (2, 4, 6), or truncations (3, 5, 7) of the *IaaH*, *IaaM*, and *Ipt* promoters upstream of the yeast *HIS3* reporter gene, and truncation of oncogene promoter. (B) Growth of the yeast strains on SD/-Ura (Synthetic Defined medium without Uracil) or SD/-His (Synthetic Defined medium without histidine) selection plates lacking 3-amino-1,2,4-triazole (3-AT) inhitor.

3.5.2 Application of the protoplast trans-activation system identified three WRKYs and ARF5 as regulators of oncogene promoter activity

Since the Y-1-H assay failed to identify transcription factors that could activate the oncogene promoters, the high-throughput protoplast transactivation (PTA) system was used (2.12). The full length of *IaaH* (337 bp), *IaaM* (337 bp) and *Ipt* (697 bp) promoters and 5' untranslated regions (5' UTR) of pTiC58 oncogenes were fused with the CDS of firefly luciferase as reporter gene (luc+/pSAT-2221, 6.2 Figure S1). A library containing the CDS of more than 400 transcription factors was tested, including members of the WRKY, AP2/ERF, bHLH, bZIP, DOF, MYB and NAC families.

The transcription factor containing expression plasmids (pHBLT 6.2 Figure S2) were co-transformed with the plasmids harboring the promoter-luciferase fusions into *Arabidopsis* mesophyll protoplast. WRKY18, WRKY40, WRKY60 and ARF5 (also named as MONOPTORUS, MP) were found to activate the *Ipt* promoter by showing enhanced reporter gene expression compared to the control protoplasts transformed which were only transformed with the *Ipt*-promoter-luciferase plasmid (Figure 14A). In contrast, no transcription factor was found to activate the *IaaH* and *IaaM* promoters (data not shown).

In order to confirm the microarray-based expression data of the three *WRKYs* and *ARF5* genes in crown gall tissues infected with *A. tumefaciens* strain C58, the transcript numbers of *WRKY18*, *WRKY40*, *WRKY60* and *ARF5* were determined by quantitative real-time PCR (2.6.7). The transcript levels in crown gall tumor were compared to those in inflorescence stems, which were inoculated by the disarmed *A. tumefaciens* strain GV3101. The transcript levels of the three *WRKY* and *ARF5* genes were higher in tumors than in stems (Figure 14B). This result was in agreement with the former microarray data [28,174].

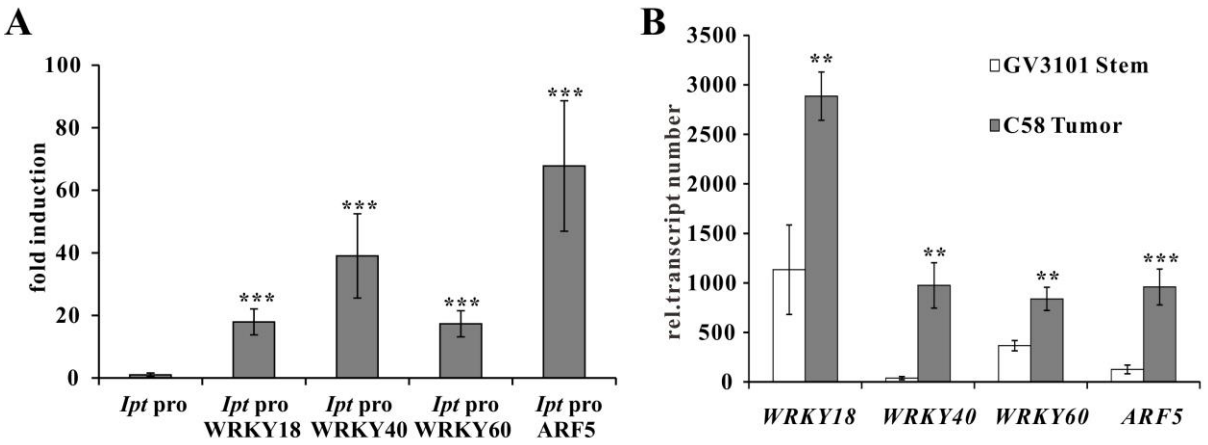


Figure 14. The WRKY18, WRKY40, WRKY60 and ARF5 transcription factor genes are expressed in crown gall tumors and activate the *Ipt* promoter of of *A. tumefaciens* strain C58.
(A) Fold induction of *Ipt* promoter-driven luminescence (*Ipt* pro) by the three WRKY transcription factors (WRKY18, WRKY40 and WRKY60) and ARF5 in *Arabidopsis* mesophyll protoplasts. The relative luminescence induced by the *Ipt* promoter in the absence of a transcription factor harboring plasmid was set to 1. Bars show mean values (±SD) of three independent experiments. (B) Relative transcript numbers of *WRKY18*, *WRKY40*, *WRKY60* and *ARF5* in crown gall tumors 25 days after inoculation of *A. tumefaciens* strain C58 (C58 Tumor) and strain (GV3101 Stems) lacking a T-DNA. Relative transcript numbers were quantified by qRT-PCR and normalized to 10,000 molecules of *ACTIN2/8*. Bars show mean values (±SD) of three independent samples. ***P<0.01* *** *P<0.001* (Student's t-test).

WRKY and ARF transcription factors bind specific DNA elements named W-box (TGAC) and AuxRE (TGTCNC or TGTCTN), respectively. There are seven W-boxes, of which one is localized in the 5' UTR, and five AuxREs in the IGR2 of pTiC58 oncogenes. Sequence comparisons of the IGR1 and IGR2 region of other Ti plasmids of *A. tumefaciens* revealed also the presence of W-boxes and AuxREs (Table 8). Like in pTiC58, the majority of them are in IGR2 of the different Ti plasmids and

only 1 or 2 of each of them in IGR1. This result suggests that the *Ipt* oncogenes, but most likely not *IaaH* and *IaaM* in the different *A. tumefaciens* strains may also be regulated by WRKY and ARF transcription factors.

Table 8 Number of W-boxes and AuxREs within the intergenic regions (IGRs) of in the different Ti plasmids of *A. tumefaciens*.

		IGR1[a]		IGR2[b]	
	pTi	W-box (TGAC)	AuxRE (TGTCNC or TGTCTN)	W-box (TGAC)	AuxRE (TGTCNC or TGTCTN)
Nopaline	pTiC58	1	1	7	5
	pTiT37	?	?	5	5
	pTiSAKURA	2	1	6	5
Octopine	pTi15955	2	1	7	5
	pTiA6NC	2	1	7	5
	pTiAch5	2	1	7	5
Agropine	pTiBo542	2	1	6	3

[a] IGR1 is localized between the coding sequences of *IaaH* and *IaaM*, [b] IGR2 is localized between the coding sequences of *IaaM* and *Ipt.*

3.6 *WRKY40* and *WRKY60* genes, but not *WRKY18* and *ARF5*, respond to *Agrobacterium tumefaciens* infections

It is known that the *WRKY18*, *WRKY40* and *WRKY60* genes are early induced upon bacterial and fungal pathogen infections [123,180]. In order to analyze the impact of *A. tumefaciens* on gene induction, the time-dependent expressions of *WRKY18*, *WRKY40* and *WRKY60* genes were analyzed in *A. tumefaciens* infected and buffer infiltrated leaf tissues by using qRT-PCR (2.6.7). The three *WRKY* genes responded to the infiltrated buffer solution at all analyzed time points (2 to 72 h) and indicate a kind of wound response (Figure 15). The transcript level of the WRKY18 was not very significantly increased at the early time points when inoculated with *A. tumefaciens*. But the transcript levels of the *WRKY40* and *WRKY60* genes were in general significantly higher within 72 hpi when inoculated with *A. tumefaciens*. Elevated levels of *WRKY40* were already observed within 2 hpi (Figure 15). A significant transcript increase of *WRKY60* was observed within 4 hpi, which peaked at 24 and 48 hpi (Figure 15). When inoculating the disarmed *A. tumefaciens* strain GV3101, which lacks a T-DNA, *WRKY18*, *WRKY40* and *WRKY60* expression were also induced within 72 hpi in a similar manner as when the virulent strain C58 was inoculated (Figure 15). In contrast, transcription of the *ARF5* gene was very low within 72 hpi and therefore not responsive to the treatment with *agrobacteria* or wounding at the time analyzed (2h to 72h, Figure 15). The gene expression results show that WRKY18 responds to wounding at the early time points and to *A. tumefaciens* infection only at 72 hpi, whereas WRKY40 and WRKY60 respond to *A. tumefaciens* at early time points of infections when the T-DNA is not yet transferred or integrated (2, 4, 8 hpi) into the host genome.

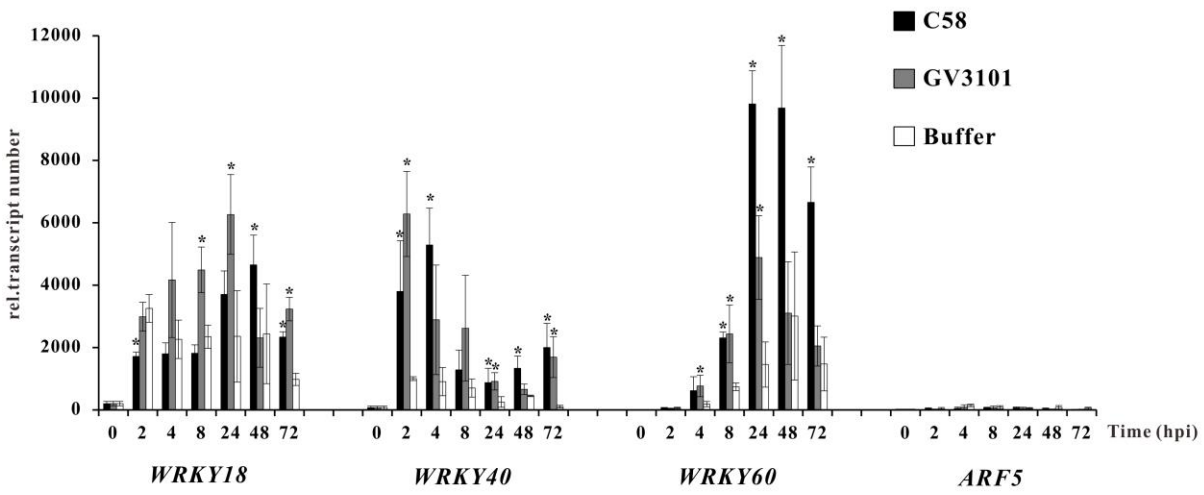


Figure 15. Time-dependent expression of the *WRKY18*, *WRKY40*, *WRKY60* and *ARF5* genes upon infection with *A. tumefaciens* strain C58, strain GV3101 and wounding.
Leaves of five-week-old *Arabidopsis* (Col-0) were infiltrated with *A. tumefaciens* strain C58, GV3101 of OD_{600} 1.0 or agromix buffer as control. Relative transcript numbers were quantified by qRT-PCR and normalized to 10,000 molecules of *ACTIN2/8*. Bars show mean values (±SD) of three independent samples. * *P<0.05* (Student's t-test).

3.7 WRKY18, WRKY40 and WRKY60 mutants display an impaired crown gall development

To characterize the role of WRKY18, WRKY40 and WRKY60 function in *A. tumefaciens*-mediated crown gall development, the *wrky* mutants were inoculated with the tumorigenic *A. tumefaciens* strain C58 (2.4.1) and the crown gall tumor weights were determined 25 days after inoculation. The three single mutants *wrky18*, *wrky40*, and *wrky60* developed smaller crown gall tumors than the wild-type plants, with *wrky18* developing the smallest tumors (Figure 16A). The two double mutants, *wrky18/wrky40* and *wrky18/60* developed even smaller tumors than the three single ones. The tumors of the *wrky40/60* double mutant were smaller than those of wild-type plants, but still bigger than the ones of the single mutants (Figure 16A). The triple mutant *wrky18/40/60* was most resistant to crown gall development and had the smallest tumors compared with the other *Arabidopsis* genotypes (Figure 16A, B). About 30% of the *wrky18/40/60* triple mutant plants did not development any crown gall tumors at all 25 days after inoculations. These findings imply that WRKY18, WRKY40 and WRKY60 transcription factors cooperatively support development of crown galls on *Arabidopsis*.

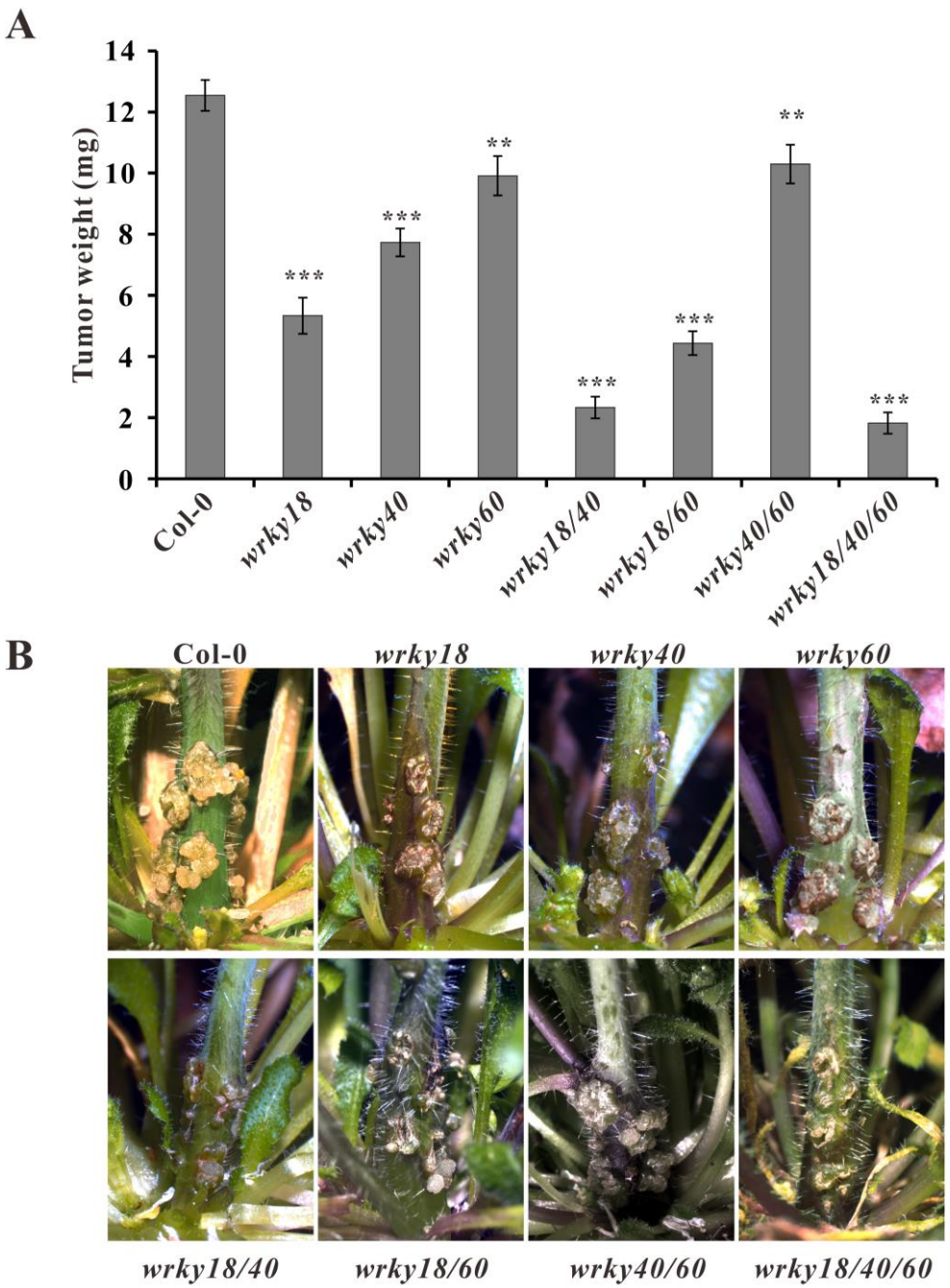


Figure 16. The *Arabidopsis wrky* mutants develop smaller crown gall tumors.
(A) Crown gall tumor weights of the *wrky* mutant and wild type plants 25 days after inoculation of the *A. tumefaciens* tumorigenic strain C58. Tumor material was weighed after separation from stems using a scalpel and a dissecting microscope. Bars show mean values of tumor weights (±SE) of at least 40 plants of each genotype. ***P<0.01*; *** *P<0.001* (Student's t-test). (B) Representative pictures of stems of each *Arabidopsis* genotype 25 days after inoculation of *A. tumefaciens* strain C58.

In order to prove the effect of WRKY18, WRKY40 and WRKY60 on *Ipt* oncogene expression, the relative transcript numbers of the *Ipt* oncogene was quantified in crown galls of *wrky* mutants using quantitative RT-PCR (Figure 17A). Compared to the wild-type plants (Col-0) with normal crown gall size, the expression of *Ipt* was not significantly changed in the smaller galls *wrky18*, *wrky40* and *wrky60* mutants. This result shows that crown gall tumors of the single *wrky* mutants do not have an

obvious effect on *Ipt* expression. Because the triple mutant (*wrky18/40/60*) developed not enough material for studying *Ipt* expression, stems samples 6 dpi were analyzed. Under these conditions, the *Ipt* transcript number was slightly, but also not significantly reduced in the triple mutant (Figure 17B). Since it is known that crown gall development requires induction of expression the three oncogenes, material gained from T-DNA-transformed tissues seems not suitable to study the impact of transcription factors on oncogene expression.

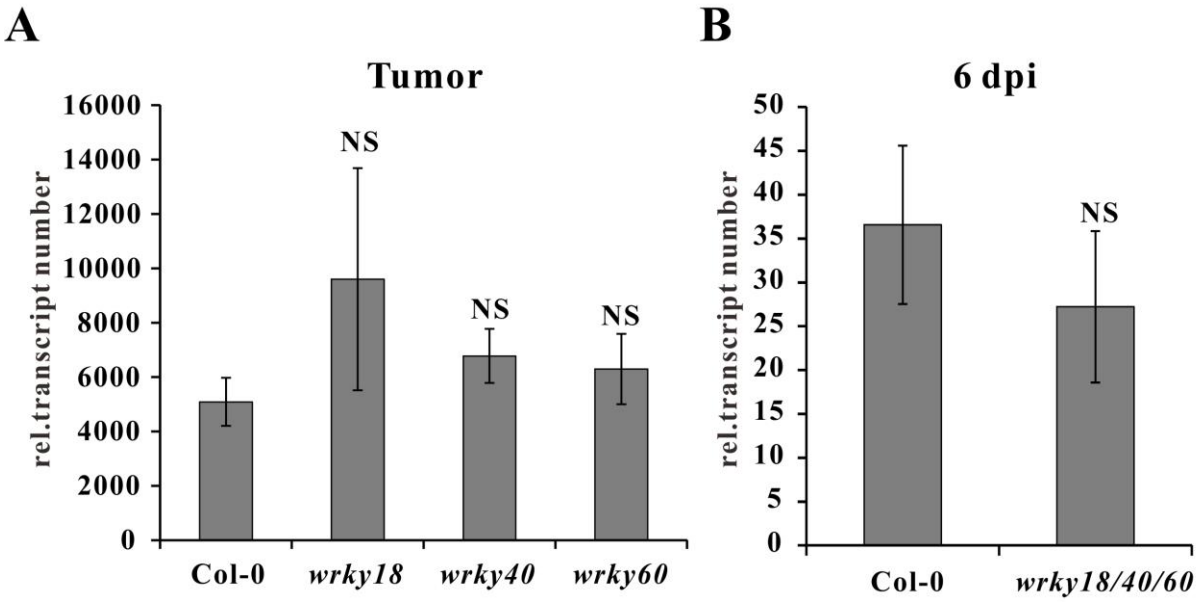


Figure 17. *Ipt* oncogene expression in *Arabidopsis* crown galls and stems six days post infection of wild-type plants and the *wrky* mutants.
(A). Relative transcript numbers of *Ipt* oncogenes in crown gall of wild-type plants and *wrky18*, *wrky40* and *wrky60* single mutant 25 days after inoculation of *A. tumefaciens* strain C58. (B). Relative transcript numbers of *Ipt* oncogenes in the stem of wild-type plants and *wrky18/40/60* triple mutant 6 days postinoculation (6 dpi) of *A. tumefaciens* strain C58. Relative transcript numbers were quantified by qRT-PCR and normalized to 10,000 molecules of *ACTIN2/8*. Bars show mean values (±SD) of three independent samples. NS: not significant.

3.8 The WRKYs interact with ARF5 and this interaction potentiates *Ipt* promoter activity

The PTA data (Figure 14A) have shown that the *Ipt* promoter can be activated by WRKY18, WRKY40, WRKY60 and ARF5. To test if these transcription factors interact with each other to cooperatively regulate the *Ipt* promoter, the Bimolecular Fluorescence Complementation (BiFC) assay (2.13) was used in *Arabidopsis* mesophyll protoplasts. The C-terminal half of yellow fluorescent protein (cYFP) was fused to the C-terminus of ARF5 to express an ARF5-cYFP fusion protein. The N-terminal half of YFP (nYFP) was also fused to the C-terminus of ARF5 as well as to the three WRKYs to generate ARF5-nYFP and WRKY-nYFP. The nYFP and cYFP fusion proteins were co-expressed in the *Arabidopsis* mesophyll protoplasts. The observation of YFP-mediated fluorescence with the confocal laser scanning microscope indicated that ARF5 interacts with itself and also with WRKY18, WRKY40 and WRKY60 (Figure 18A). The fluorescence was specifically localized in the

plant nucleus. When, WRKY40-cYFP was co-expressed with ARF5-nYFP and with the WRKYs fused to nYFP in the protoplasts, YFP fluorescence was also observed in the nucleus (Figure 18B). The free cYFP construct was used as negative control, and showed no YFP fluorescence when co-expressed with the WRKY-nYFPs and ARF5-nYFP in the protoplasts (Figure 18C).

It has been reported that the domain III and IV at the C-terminus of ARF5 is important for dimerization and protein-protein-interaction [132,181,182]. To test, if these domains are required for the interaction with WRKYs, the C-terminal deletion of ARF5 (1–722 aa), ARF5Δ722-cYFP was co-expressed with either ARF5-nYFP or the three WRKY-nYFPs. The truncated ARF5Δ722 protein was unable to interact with the intact ARF5 protein or with WRKY18, WRKY40 and WRKY60 (Figure 18D). This result indicates that the domains III and IV are also important for interaction with the three WRKYs. In order to check whether the interactions between ARF5 and WRKY18, WRKY40, WRKY60 are specific, ARF3-cYFP, which naturally lacks domain III and IV and WRKY53-cYFP, which was also induced at early time points of *A. tumefaciens* infections (3 hpi; 6.1 Table S1) were tested for interacting with the three WRKYs and ARF5 [28,132]. Both ARF3 and WRKY53 did not interact with ARF5, WRKY18, WRKY40, and WRKY60 and proved that the interaction between the WRKYs and ARF5 is specific (Figure 18E, F).

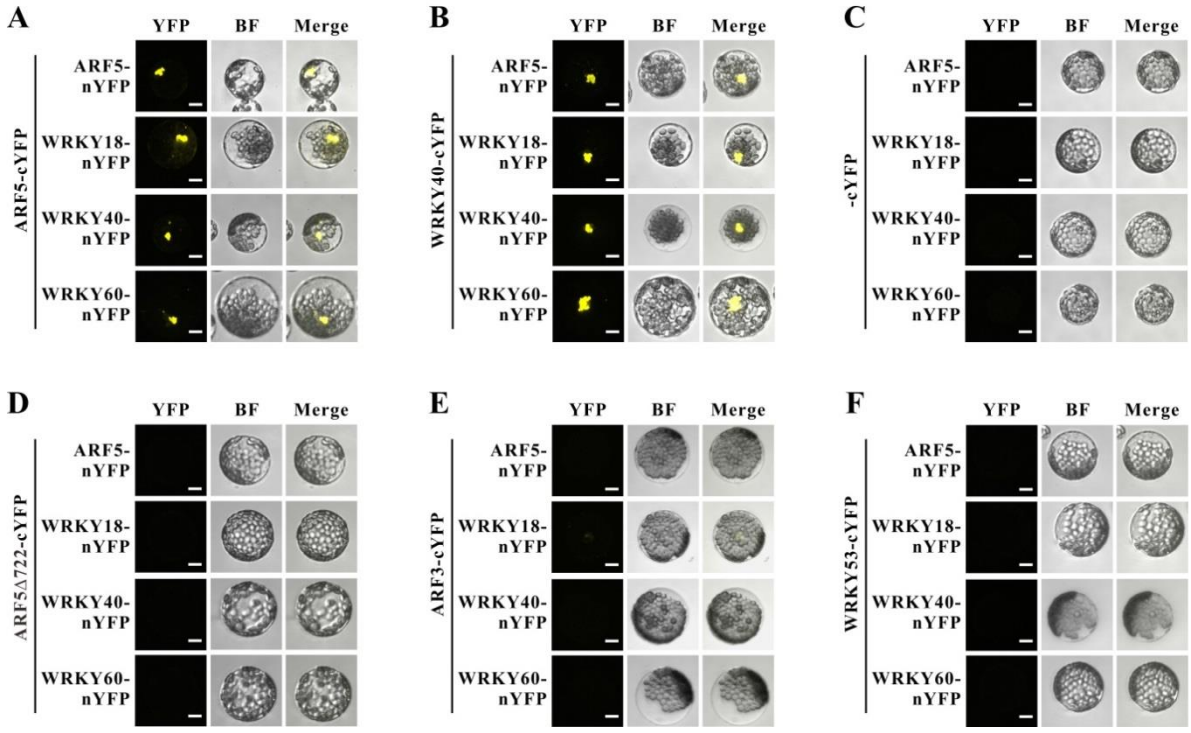


Figure 18. The three WRKYs interact with ARF5 and itself in the nucleus in a bimolecular fluorescence complementation (BiFC) assay in *Arabidopsis* mesophyll protoplasts.
(A) ARF5-cYFP, (B) WRKY40-cYFP, (C) cYFP, (D) the C-terminal deletion version of ARF5, ARF5Δ722-cYFP, (E) ARF3-cYFP and (F) WRKY53-cYFP were co-expressed with ARF5-nYFP, WRKY18-nYFP, WRKY40-nYFP and WRKY60-nYFP in *Arabidopsis* mesophyll protoplasts. YFP: yellow fluorescence protein; BF: bright field; Merge: merged pictures of the YFP and the corresponding bright field. Bars = 10 µm.

The PTA system was used, to study the impact of the cooperation between ARF5 and the three WRKYs on *Ipt* promoter activity. When WRKY40 was co-expressed with ARF5 in *Arabidopsis* mesophyll protoplasts, the induction of luciferase expression driven by the *Ipt* promoter was significantly higher in the presence of ARF5 and WRKY40 compared to ARF5 or WRKY40 alone (Figure 19). In contrast, expression of ARF5 together with WRKY18 or with WRKY60 did not enhance the *Ipt* promoter activity to induce luciferase-catalyzed luminescence (Figure 19). These results indicated that WRKY40 is more important than WRKY18 and WRKY60 for the activation of *Ipt* promoter.

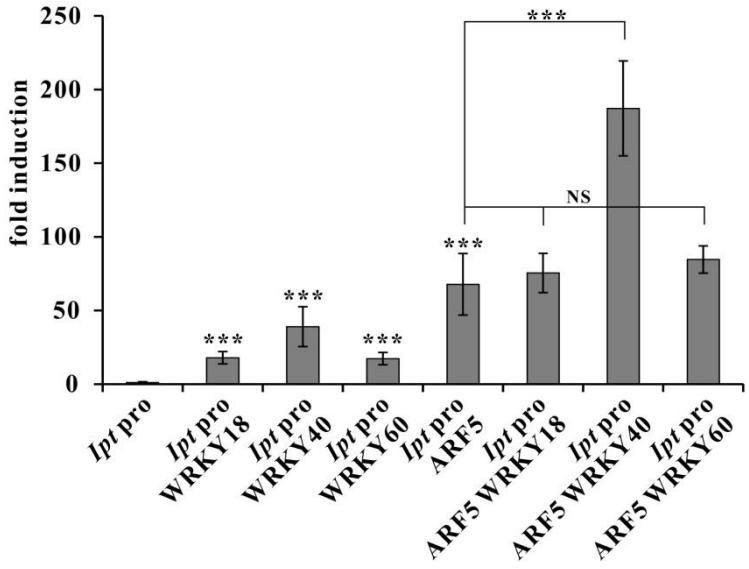


Figure 19. Co-expression of the three WRKYs and ARF5 together with the *Ipt* promoter in the protoplast transactivation system.
Fold induction of *Ipt* promoter (*Ipt*-pro)-driven luminescence in *Arabidopsis* mesophyll protoplasts. The relative activity of the *Ipt* promoter in protoplast without transformation of any of the transcription factor constructs was set to 1. ARF5 was co-expressed with WRKY18, WRKY40 or WRKY60. Bars show mean values (±SD) of three independent experiments *** $P<0.01$ (Student's t-test). NS: not significant.

3.9 WRKY40 directly binds to the *Ipt* promoter

The results of the PTA assay indicate that WRKY40 has a stronger effect on activation of the *Ipt* promoter than WRKY18 and WRKY60, suggesting that WRKY40 might regulate the *Ipt* promoter directly. To prove the binding of WRKY40 to the *Ipt* promoter, the electrophoretic mobility shift assays (EMSA, 2.11) was performed. The full length CDS of WRKY40 including the stop codon was cloned into pET28b to express a fusion protein with six histidine tagged to the N-terminus of the WRKY40 protein (2.8.1). The 6×His::WRKY40 fusion protein was induced by IPTG and expressed in the *E. coli* strain SoluBL21 (2.9.1; Figure 20A). The Western blot result showed that the 6×His::WRKY40 protein fusion was detected in the total protein extract (lane T), the soluble (lane S) and insoluble cell fraction (lane P; Figure 20A). The tagged protein was finally purified from the supernatant and used in the EMSA (2.9; lane E, Figure 20A). A 50 bp fragment (−184 bp to −135 bp) of the *Ipt* prompter which contains three of the six W-boxes located in the promoter region, was

radioactively labeled (2.9) and served as probe for EMSA (Figure 20B). A very weak band shift (WRKY40-*Ipt* complex) was observed in the presence of 150 ng purified recombinant 6×His::WRKY40 protein, but the double amount of the tagged WRKY40 protein (300 ng) showed a much stronger band (Figure 20C). The addition of unlabeled *Ipt* promoter element as competitor to the reaction mixture significantly reduced the binding of WRKY40 to the labeled *Ipt* promoter probe. Thus, WRKY40 binds to the *Ipt* probe *in vitro* and the *Ipt* promoter therefore might be a direct target of the WRKY40 in plant cells.

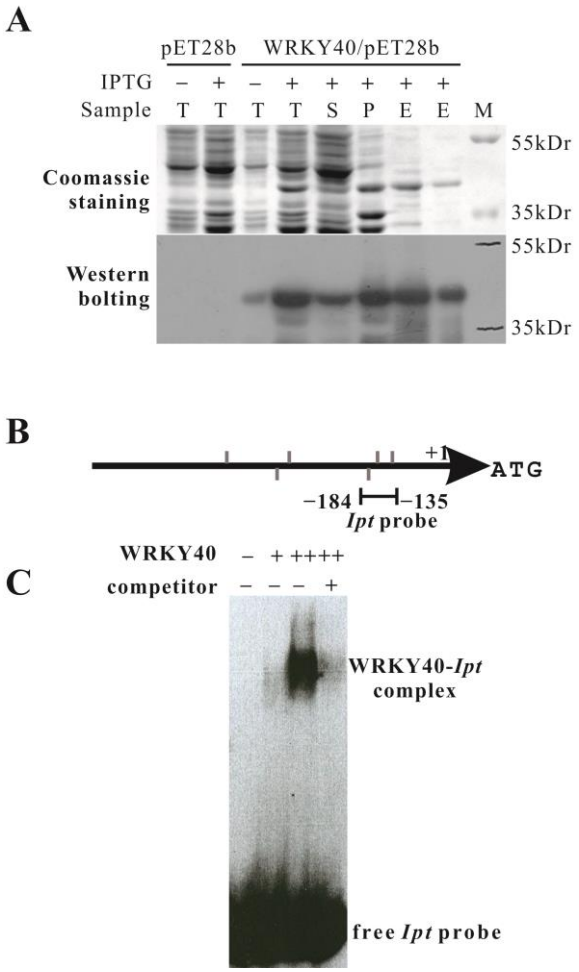


Figure 20. WRKY40 binds to the *Ipt* promoter *in vitro*.
(A) A Coomassie stained gel and Western blot of protein extracts from *the E. coli* strains, harboring either the empty vector pET28b or the recombinant plasmid WRKY40/pET28b. The *E. coli* cells were cultivated in the presence (+) or absence (-) of IPTG. A monoclonal anti-histidine antibody (5 PRIME, Hamburg, Germany) was used as primary antibody to detect the 6x histidine-tagged WRKY40 protein. T: total protein; S: Supernatant and P: Pellet after sonication; E: Eluted purified protein; M: protein marker. (B) Positions of W-boxes (TGAC, grey bars) in the sense (above the line) and anti-sense strand (below the line) of the *Ipt* promoter. The line below the promoter (−184 bp to −135 bp) indicates the fragment used as *Ipt* probe in EMSA. (C) Electrophoresis mobility shift assay (EMSA). WRKY40 (-) indicates in the absence and (+) in the presence of 150 ng or (++) 300 ng purified recombinant histidine-tagged WRKY40 protein. Competitor (-) indicates without and (+) with addition of unlabeled probe.

3.10 Impact of phytohormone signaling pathways on oncogene promoter activity

Previous studies have shown that the levels of free IAA in infected *Arabidopsis* tissues are increased more than two-fold six days after inoculation of *A. tumefaciens* compared to mock-treated plants, and crown gall tumors accumulated more than four-fold free IAA compared to control tissues [28]. In addition, the total levels of cytokinin in *Arabidopsis* crown gall tissues of *A. tumefaciens* strain C58 is 8414 ng/g (dry weight) compared to 849 ng/g (dry weight) in the tumor-free stem tissues. The dominant cytokinin form in *Arabidopsis* crown gall tissue is zeatin (544 ng/g) and its conjugates, such as zeatin riboside (2294 ng/g) and zeatin nucleotide (3657 ng/g).

3.10.1 Auxin, but not cytokinin induces oncogenes expression

Because of the elevated levels of auxin and cytokinin in T-DNA transformed cells, the PTA system (2.12) was used to analyze the impact of auxin and cytokinin on the *IaaH, IaaM* and *Ipt* promoter activity. The *Ipt* promoter was highly induced by the bioactive auxin type 1-NAA, and also by the cytokinin type *trans*-zeatin (Figure 21). However, trans-zeatin was much less effective than 1-NAA. The increasing concentrations of auxin and cytokinin had no strongly increasing effect on activity of the three oncogene promoters (Figure 21).

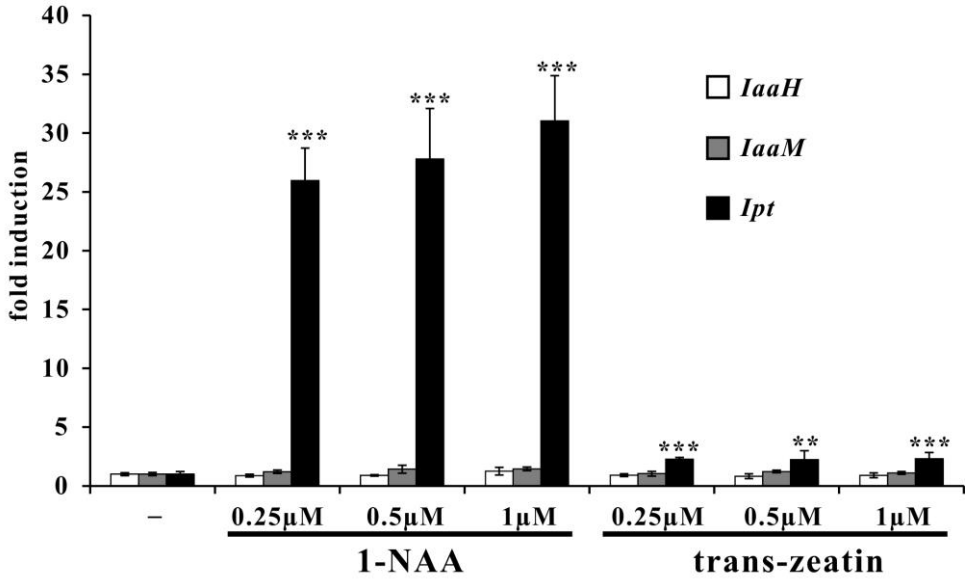


Figure 21. Effect of auxin (1-NAA) and cytokinin (*trans*-zeatin) on the *IaaH*, *IaaM*, and *Ipt* promoter activity.
Fold inductions of oncogene promoter-driven luminescence in *Arabidopsis* mesophyll protoplasts treated with auxin (1-NAA) or cytokinin (trans-zeatin) in different concentrations. The protoplasts had been transformed with the *IaaH*, *IaaM*, and *Ipt* promoter-luciferase reporter gene constructs before they were incubated with the different concentration of 1-NAA or *trans*-zeatin. The relative activity of the luciferase reporter in protoplasts without phytohormone treatment was set to 1. Bars show mean values (±SD) of three independent experiments. *** $P<0.01$ (Student's t-test).

3.10.2 ARR1 and ARR4 do not activate the *IaaH, IaaM*, and *Ipt* promoters

The oncogene promoters of *IaaH, IaaM* and *Ipt*, contain ARR1 binding elements (GATT; Table 7), suggesting that the three oncogenes might be regulated by the type B ARR transcription factors, which

mediate cytokinin signaling. The ARR1 gene was low expressed in crown gall tumor tissue infected with the virulent *A. tumefaciens* strain C58 compared to stems infected with disarmed strain GV3101. Instead another type B transcription factor ARR4 was strongly expressed in crown gall tumors (Figure 22A). The ability of the ARR1 and ARR4 transcription factors to activate the *IaaH*, *IaaM*, *Ipt* promoters was tested in the PTA system. ARR1 and ARR4 did not significantly increase luciferase activity driven by the three oncogene promoters, also not in the presence of *trans*-zeatin (Figure 22B). These results suggested that cytokinin signaling is not a key player to regulate *IaaH*, *IaaM* and *Ipt* expression.

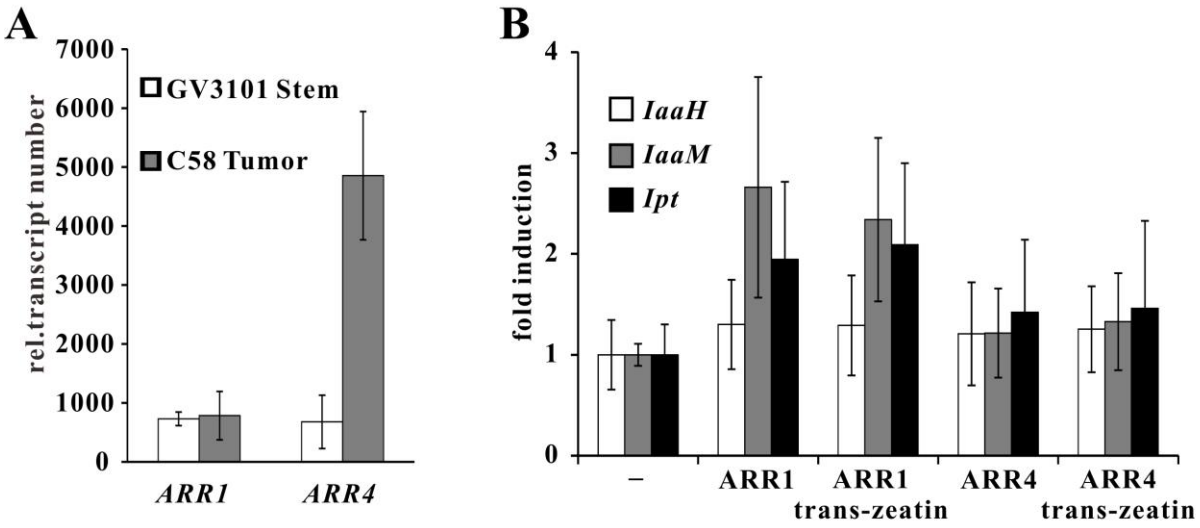


Figure 22. ARR1 and ARR4 do not activate the oncogene promoters.
(A) Relative transcript numbers of the *ARR1* and *ARR4* genes in crown gall tumors 25 days after inoculation with the virulent A. tumefaciens strain C58 (C58 Tumor) and in stems inoculated with the disarmed strain GV3101 (GV3101 Stems). Relative transcript numbers were quantified by qRT-PCR and normalized to 10,000 molecules of *ACTIN2/8*. Bars show mean values (±SD) of three independent samples. (B) Fold induction of *IaaH, IaaM, Ipt* promoter-driven luminescence in *Arabidopsis* mesophyll protoplasts transformed with ARR1 and ARR4 constructs and in the presence or absence of *trans*-zeatin. The relative activity of the luciferase reporter construct in the absence of ARRI, ARR4 transcription factors and trans-zeatin was set to 1. Bars show mean values (±SD) of three independent experiments. This experiment was performed by Fabian Imdahl (Bachelor student of Julius-von-Sachs-Institute, Department of Molecular Plant Physiology and Biophysics, University of Wuerzburg, Germany).

3.10.3 ARF5 activates the *Ipt* promoter in an auxin-dependent manner

The auxin type 1-NAA and the ARF5 transcription factor strongly activate the *Ipt* promoter, but not those of *IaaH* and *IaaM*. (Figure 14A, 21). In addition, the *Ipt* promoter sequence contains five auxin response elements (AuxREs, TGTCNC or TGTCTN) for binding of ARF transcription factors (Table 7), which are absent in the *IaaH* and *IaaM* promoter sequences. ARF transcription factors usually regulate their target gene in an auxin-dependent manner [149,150]. Therefore, the regulatory effect of ARF5 on the *Ipt* promoter was analyzed in the presence of auxin in the PTA system (2.12). ARF5 activated the *Ipt* promoter, and this activation was even stronger when the protoplasts were treated with auxin (1-NAA, Figure 23). It is known that auxin/indole-3-acetic acid (Aux/IAA) proteins can inhibit the ARF transcription factor-dependent promoter activation. The inhibitor of ARF5 is IAA12

(also named as BODENLOS, BDL) [181]. When *Arabidopsis* mesophyll protoplasts were co-transformed with ARF5 and IAA12 plasmid constructs, the *Ipt* promoter activity, which drives luciferase expression, was significantly reduced compared to the ones transformed with ARF5 alone (Figure 23). However, the level of the *Ipt* promoter activity was not as low as it was in the absence of any transcription factor, which may indicate that not all ARF5 proteins are inhibited by IAA12. This is because the protoplasts were transformed with equal amount of ARF5 and IAA12 expression plasmid, but not equal amount of proteins.

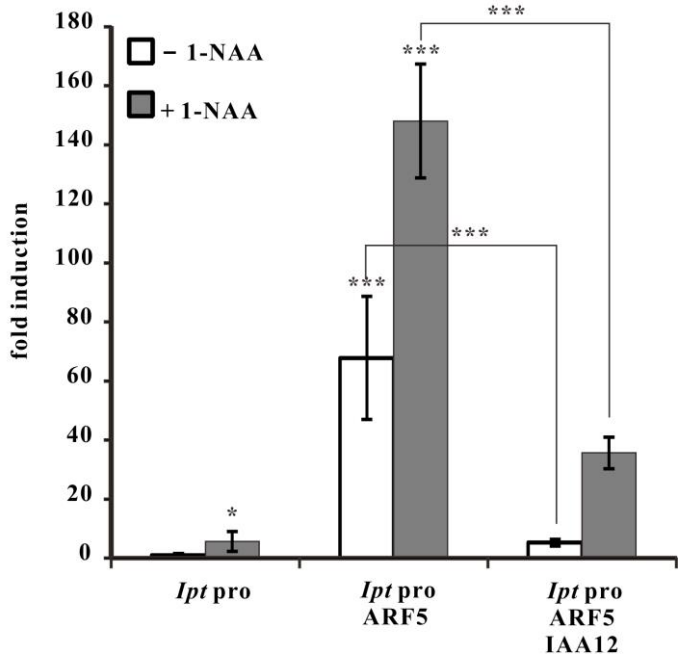


Figure 23. The transcription factor ARF5 activates the *Ipt* promoter in an auxin-dependent manner.
Fold induction of *Ipt* promoter-driven luminescence in *Arabidopsis* mesophyll protoplasts (*Ipt* pro) in the presence or absence of the transcription factors ARF5 and IAA12 as well as in the presence (+ 1-NAA) and absence (- 1-NAA) of auxin. The relative luminescence induced by the *Ipt* promoter in the absence of transcription factors and auxin treatment was set to 1. Bars show mean values (±SD) of three independent experiments. * *P<0.05*; ** *P<0.01*; *** *P<0.001* (Student's t-test).

There are five AuxREs located in the *Ipt* promoter sequence, two in sense and three in antisense direction (Figure 24A). Each of these AuxREs were individually mutated (TGTCNC to TGGCNC and TGTCTN to TGGCTN; 1m, 2m, 3m, 4m, 5m) as well as all of them together (1-5m). These mutated full-length promoters were tested in the PTA assay. The single mutated AuxREs caused a reduction in ARF5- and auxin-dependent *Ipt* promoter activity (ARF5 + 1-NAA), which was however not completely abolished (Figure 24B). Mutating all five AuxREs (1-5m) totally abolished the activation of the *Ipt* promoter after auxin treatment in the absence and presence of ARF5 (Figure 24B). In addition, the fifth mutated AuxRE (5m) had the more reduction effect compared to the other AuxREs (1-4m), which indicated the fifth AuxRE localized in *Ipt* element seemed to be the motst important AuxRE. When the *Ipt* promoter fragment from −150 (from the TSS) to the translational start codon (ATG), containing the conserved region (*Ipt* element, Figure 24A) with two AuxREs, was analyzed in

the PTA system, ARF5 activated the truncated *Ipt* promoter. Nonetheless the activation was not as strong as with the full length *Ipt* promoter (Figure 23, 24C). In addition, the *Ipt* element from −150 to −91 in the *Ipt* promoter (Figure 24A) was fused with the 35S minimal promoter and also analyzed in the PTA system. The *Ipt* element mediated a similar level of luminescence induction as the truncated Ipt −150 promoter (Figure 24D). These results suggest the full length of Ipt promoter and all five AuxREs are necessary for full activity of Ipt promoter activated by ARF5 and auxin.

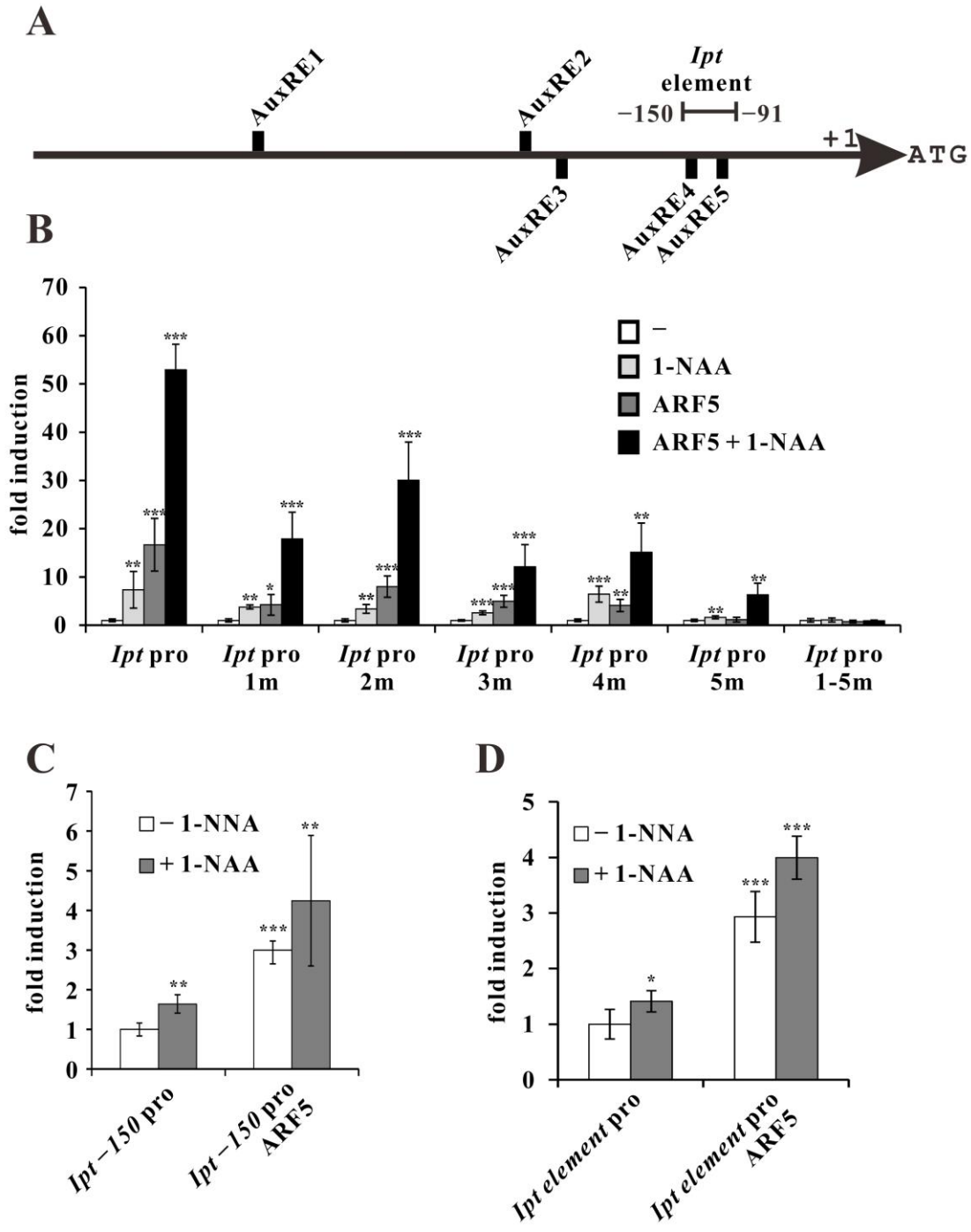


Figure 24. Auxin-dependent regulation of the *Ipt* promoter by ARF5.
(A) Positions of the auxin responsive elements (AuxREs; black bars, TGTCNC or TGTCTN) on the sense (above the line) and antisense strand (below the line) and the transcription start site (TSS +1) within the *Ipt*

promoter. (B) Fold induction of *Ipt* promoter-driven luminescence activity in transformed *Arabidopsis* mesophyll protoplasts when the five AuxREs are mutated (AuxREm: TGTCNC to TGGCNC and TGTCTN to TGGCTN). (C) Fold induction of a truncated *Ipt* promoter-driven luminescence (–150 bp from TSS; *Ipt* –150 pro) in *Arabidopsis* mesophyll protoplasts. (D) Fold induction of the *Ipt* element promoter-driven luminescence in *Arabidopsis* mesophyll protoplasts. The *Ipt* element was fused with 47 bp of the CaMV35S minimal promoter. The activity of the different Ipt promoter constructs was determined by adding auxin (+ 1-NAA) or omitting it (- 1-NAA) in the presence or absence of ARF5. The relative activity of the Ipt promoter-luciferase reporter construct in the absence of any transcription factors or auxin treatment was set to 1. Bars show average values (±SD) of three independent experiments. * *P<0.05*; ** *P<0.01*; *** *P<0.001* (Student's t-test).

3.10.4 The auxin receptor TIR1 is required for normal crown gall development

Because of the strong growth phenotype of the *arf5/mp* mutant plants [138,139], it is not possible to study the crown gall development on *these mutants*. However, the data of this study show that ARF5 regulates the *Ipt* promoter activity in an auxin-dependent manner, which suggest a role for the auxin signaling pathway in regulation of *Ipt* expression. It is known that the auxin receptor, transport inhibitor response 1 (TIR1) protein, which is a part of SCF^{TIR} ubiquitin ligase complex promotes Aux/IAA protein degradation at high auxin concentrations which releases the ARF transcription factors and allows them to bind to AuxREs and activate auxin sensitive promoters. Therefore, the *tir1* mutant was chosen to study its impact on crown gall tumor development. The tumor weight of the *tir1* mutant (*tir1-1*) was determined 25 days after inoculation of the virulent *A. tumefaciens* strain C58 into stems. The *tir1* mutant developed smaller tumors than the wild-type plants (Figure 25A, B). This result supports the idea that auxin signaling is required for normal crown gall tumor development.

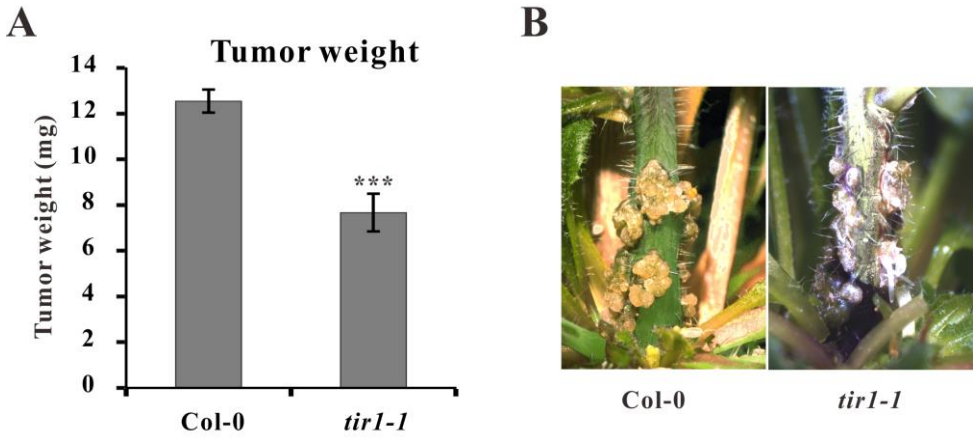


Figure 25. The *Arabidopsis tir1-1* mutant develops smaller crown gall tumors.
(A) Crown gall tumor weights of the *tir1-1* mutant and wild type Col-0 plants 25 days after inoculation of *A. tumefaciens* strain C58. Tumor material was weighed after separation from the inoculated stems using a scalpel and dissecting microscope. Bars show mean values of tumor weights (±SE) of at least 40 plants of each genotype. ***P<0.01*; *** *P<0.001* (Student's t-test). (B) Representative pictures of stems of the two *Arabidopsis* genotypes 25 days after inoculation of the virulent *A. tumefaciens* strain C58.

4. Discussion

As a plant pathogen, *Agrobacterium tumefaciens* utilizes the mechanism of gene transcription in host cells to transcribe its own T-DNA encoded oncogenes, *IaaH*, *IaaM* and *Ipt*. The expression of the oncogenes results in overproduction of the phytohormones auxin and cytokinin, which induce uncontrolled cell proliferation and crown gall development. The *A. tumefaciens*-mediated plant cell transformation process and the roles of the oncogene encoded enzymes have been well studied [9,10,19], but regulation of oncogene expression in plant cells is still not well understood. Therefore, this study attempted to answer the question, how the expression of the *IaaH*, *IaaM* and *Ipt* oncogenes is regulated in the host cell.

4.1 The oncogenes possess pro- and eukaryotic features for transcription in pro- and eukaryotic cell types

A group of T-DNA encoded genes of the *A. tumefaciens* Ti plasmids is responsible for plant crown gall development and called oncogenes. The most essential oncogenes for crown gall development are the *IaaH, IaaM and Ipt* genes (1.2) and it is well established that they are transcribed in plant cells (Figure 6B) [28]. The coding sequences (CDS) of the three oncogenes are separated by two non-coding intergenic regions (IGR1 and IGR2; Figure 7A). The IGRs function as promoters to induce transcription of the *IaaH*, *IaaM* and *Ipt* oncogenes in plant cells (Figure 7B). Eukaryotic cells require promoter sequence elements for transcriptional initiation that are recognized by a complex composed of the general transcription factors and RNA polymerase II. These common sequence motives are the transcription start site (TSS) and the TATA box [55]. In this study, the eukaryotic TSSs and *cis*-regulatory motives of the agrobacterial oncogenes *IaaH*, *IaaM* and *Ipt* were determined in the T-DNA of the virulence plasmid pTiC58 from *A. tumefaciens* strain C58 by using the 5' RACE assay (Table 6). The consensus sequences around the TSSs (Inr boxes, YR Rule) are in agreement with the common plant TSS [56,57]. The TATA boxes of the three oncogenes are localized in a distance from the TSS, known from actively transcribed eukaryotic genes (Table 6). In addition, CAAT boxes are also found in all three oncogene promoters (Table 6). An alignment of oncogene promoters shows that the TSSs and *cis*-regulatory motives are highly conserved in the different types of Ti plasmids (Figure 8, 9, 10). Only one TATA box is localized in *IaaH* and *IaaM* promoter. In contrast, it is reported that two TATA boxes are present 5' upstream of the CDS of the *Ipt* genes from the octopine Ti plasmids, pTiA6NC [170], pTiAch5[168,169] and pTi15955[183]. The first TATA box (TATAAA) contributes to the "short" and major transcript and the second TATA box (TATAAC) contributes to the "long" transcript [168]. In contrast, in the nopaline type Ti plasmids pTiT37 [167], pTiSAKURA, and pTiC58 as well as in the agropine Ti plasmid pTiBo542, only one TATA box (TATAAC) is present (Figure 10). In our study, only one TATA box preceded the TSS of *Ipt* gene in pTiC58, according to the 5' RACE result (Table 6). It is reported that at least one TATA box is essential for *Ipt* expression [151], and

speculated that two TATA boxes may function as an enhancer to stimulate transcription [170]. This suggests that the basic *cis*-regulatory sequences in the *Ipt* promoters vary in different types of the Ti plasmid suggesting different levels of expression of the *Ipt* gene.

Transcription of the *IaaH*, *IaaM*, and *Ipt* oncogenes in plant cells is undoubted. However, it is still not clear whether these oncogenes are also expressed in *A. tumefaciens* cells. In the 1980s, it was reported that transcripts of the T-DNA region were detected in the bacterium. At least the transcripts of twelve T-DNA regions including *IaaH*, *IaaM* and *Ipt* have been found in *A. tumefaciens* strain C58 by cDNA-DNA Southern blotting analysis [184]. Because of the limitation of the molecular biological techniques at that time, contaminations with DNA in the total RNA preparation cannot be excluded. Furthermore, three proteins (molecular weight 74 kD; 28 kD and 27 kD) were found to be expressed from the T-DNA region of pTiAch5 and pTiC58 in *E. coli* minicells, and also in *E. coli* and *A. tumefaciens* cell-free systems, but the protein products have never been identified [185]. In this study, the transcription of the three oncogenes *IaaH*, *IaaM* and *Ipt* were determined in the *A. tumefaciens* strain C58 using RT-PCR (Figure 6A). The transcripts were detected in the cDNA of *A. tumefaciens* strain C58 no matter if the culture was treated with or without acetosyringone (Figure 6A). This result shows that the agrobacterial oncogenes *IaaH*, *IaaM* and *Ipt* are transcribed in *A. tumefaciens* independently of virulence induction. For transcription initiation in prokaryotic cells, the RNA polymerase usually recognizes the −10 element (TATAAT) and −35 element (TTGACA) in prokaryotic promoters [55]. The −10 element is similar to the eukaryotic TATA box (TATAAA) present in the three oncogene promoters. Therefore, it may be speculated that these elements in the oncogene promoters may function as both prokaryotic −10 element in *A. tumefaciens* cells and as eukaryotic TATA box in plant cells. This enables *A. tumefaciens* to express the oncogenes in prokaryotic as well as in eukaryotic cell types. As the TSSs of *IaaH*, *IaaM* and *Ipt* oncogenes have not yet been identified in *A. tumefaciens* cells and the −35 elements are not found at the corresponding position upstream of −10 element, further experimental evidence is necessary to support this speculation.

4.2 *Ipt* oncogene, but not *IaaH* and *IaaM*, is regulated in an auxin-dependent manner

It is reported that *A. tumefaciens* produces auxin and cytokinin themselves [28,186], so the presence of *IaaH*, *IaaM* and *Ipt* transcripts in *A. tumefaciens* cells (Figure 6A) may participate in auxin and cytokinin biosynthesis by the bacterium. It is is known that cytokinin in mainly synthesized by two enzymes in *A. tumefaciens*: (*i*) transfer RNA (tRNA) isopentenyltransferase encoded by chromosomal gene *miaA* [187,188]; (*ii*) trans-zeatin synthesizing (tzs) enzyme encoded by *tzs* gene in the vir region of the nopaline pTi-plasmid [30,186,189]. The *tzs* gene is induced by acetosyringone, and the *tzs* enzyme catalyzes the same reaction in the cytokinin biosynthesis pathway as the *Ipt* protein

[30,186,189]. In this study the *Ipt* transcripts were determined in *A. tumefaciens* strain C58 without treatment of acetosyringone, which indicates that expression of the *Ipt* gene is independent of virulence induction (Figure 6A). Thus, *A. tumefaciens* strain C58 may synthesize cytokinin ubiquitously via the *Ipt* enzyme as well as after stimulation by the acetosyringone-induced tzs protein. Cytokinin has effects on cell division, which is essential for the proliferation of crown gall tumors. In addition, cytokinin represses a Myb transcription factor to increase the transformation of *A. tumefaciens* [31]. The effect of cytokinin on oncogene promoter activity was analyzed in the PTA system. Only the *Ipt* promoter, but not *IaaH* and *IaaM*, was induced slightly by the cytokinin type *trans*-zeatin in *Arabidopsis* mesophyll cells (Figure 21). Eight and seven binding elements for the ARR1 transcription factor are located in *IaaH*/*IaaM* bidirectional promoter and *Ipt* promoter, respectively (Table 7). ARR1 is a type-B ARR transcription factor, which activates transcription of cytokinin responsive genes [190,191]. However, all three promoters were not activated by ARR1, even in the present of *trans*-zeatin (Figure 22B). Similarly, another type-B ARR transcription factor, ARR4, which is higher expressed in crown gall tumors than in non-tumorous stem tissue, did also not induce *IaaH*, *IaaM* and *Ipt* promoter activity (Figure 22). These results indicate that cytokinin signaling does not have a dominant role in oncogene expression.

The auxin type 1-NAA was much more effective than trans-zeatin to activate the *Ipt* promoter. At least five tryptophan-dependent pathways of auxin biosynthesis are utilized by bacteria including (*i*) the indole-3-acetamide (IAM) pathway, (*ii*) the indole-3-pyruvic acid (IPA) pathway, (*iii*) the tryptamine pathway, (*iv*) the indole-3-acetonitrile (IAN) pathway and (*v*) the tryptophan side-chain oxidase (TSO) pathway [192]. The IAM pathway, mediated by the IaaH and IaaM enzymes, is important in plant cells transformed with the T-DNA of *A. tumefaciens* for crown gall development This auxin biosynthesis pathway has also been identified to be used in other phytopathogens, such as *Erwinia herbicola* pv. *Gypsophilae*, *P. savastanoi* and *Pseudomonas syringae* [18,192]. The auxin synthesized by gall-forming bacteria is beneficial for gall development [192]. As the transcripts of *IaaH* and *IaaM* are found in the virulent *A. tumefaciens* strain C58 (Figure 6A), it may be speculated that this strain also synthesizes IAA via the IAM pathway. Auxin is produced in the agrobacterial cell and can be determined in the cultivation medium [28,193], which suggests that *A. tumefaciens* secretes auxin into the apoplast. Elevated levels of free IAA in early infected *Arabidopsis* tissue is observed at six days after inoculation [28]. This combines the auxin synthesized by IaaH and IaaM enzyme in both transformed plant tissue and *A. tumefaciens*.

The results of this study show that the *Ipt* promoter, but not *IaaH* and *IaaM*, is strongly activated by auxin (Figure 21). The *Ipt* promoter contains five AuxREs (auxin response elements) and is activated by the auxin response factor ARF5 (also named as MONOPTORUS, MP) in an auxin-dependent manner (Figure 23). ARF transcription factors are essential components of the auxin signaling pathway and regulate genes, which contain AuxREs in their promoters, in combination with Aux/IAA

proteins in an auxin-dependent manner [134,149]. So auxin is a key factor regulating *Ipt* oncogene expression. Moreover, proliferation of crown gall tumor is known to be promoted in vascularized regions of inflorescence stems and veins of leaves [194]. The ARF5 transcription factor is expressed specifically in vascular tissue, where it is restricted to provascular tissues of cotyledons and hypocotyls, and the vascular bundles of inflorescence stems and veins of leaves [139,195]. Besides, *ARF5* gene expression is induced by auxin [181,195]. In conclusion, the elevated auxin levels in the *A. tumefaciens* infected and T-DNA transformed plant tissue may induce *ARF5* gene expression, which finally leads to an ARF5-dependent activation of the *Ipt* promoter. The *arf5/mp* mutant seedling fails to form normal roots and cotyledons and does not develop mature plants [138,139]. Therefore, it is not possible to study the role of *arf5/mp* in crown gall development in comparison to wild-type plants. However, the regulation of ARF5 function involves TIR1 (transport inhibitor response 1), an auxin receptor, which is an F-box protein and part of the SCF^{TIR} ubiquitin ligase complex for poly-ubiquitination of IAA proteins. In order to get an idea, if this auxin signaling pathway is relevant for crown gall development, the *tir1* mutant was analyzed instead of ARF5 in this study. The *tir1* mutant develops smaller crown gall tumors than the wild-type plants pointing to a role of the TIR1-mediated auxin response on crown gall tumor development (Figure 25).

4.3 *Agrobacterium tumefacines* recruits the pathogen defense pathway to induce *Ipt* oncogene expression and crown gall development

The single mutant plants of *wrky18, wrky40,* clearly developed smaller crown gall tumors than the wild-type plants. The tumors of double mutant *wrky18/40* and *wrky18/60* were much smaller than the single mutants. The triple mutant *wrky18/40/60* had the smallest tumors. The impaired crown gall tumor growth of *wrky60* and *wrky40/60* in *A. tumefaciens* were not as strong as that of the others (Figure 16). Nevertheless, the results suggest that WRKY18, WRKY40, and WRKY60 have a positive effect on crown gall development. It has been reported that WRKY18, WKRY40 and WRKY60 have a complex but distinct pattern of response to different kinds of pathogens [123]. The *wrky* mutants are more resistant to biotrophic pathogen, for example *Pseudomonas syringae* [123], powdery mildew *Golovinomyces orontii* [180], but more susceptible to necrotrophic pathogen *Botrytis cinerea.* [123]. For resistance to biotrophic pathogen, the single mutant *wrky18* displays resistance to *P. syringae*, and the resistance of double mutant *wrky18/40* and *wrky18/60* are much stronger than the *wrky18* single mutants. The triple mutant *wrky18/40/60* exhibits the strongest resistance to *P. syringae* [123]. As *A. tumefaciens* is also a biotrophic pathogen the pattern of defense response described for the *wrky* mutants correlates with the size of crown galls, developed on the different *wrky* mutant plants (Figure 16).

Although the three WRKY transcription factors activate the *Ipt* promoter and show impaired crown gall growth, transcription of the *Ipt* gene is not significantly changed in crown galls of the *wrky* single

mutants compared to wild-type plants (Figure 17A). The triple mutant showed a slight reduction in *Ipt* transcription which was however not significantly different to that in wild-type plants (Figure 17B). The reason for this finding is that the expression of *Ipt* was determined in crown gall tissues. Even small crown galls can only develop when the oncogenes are expressed. Therefore, inoculated or developing crown gall tissue seems not to be suitable for studying oncogene transcription. An additional reason for the normal levels of *Ipt* transcript numbers in the *wrky* mutants may be that the *ARF5* gene, which is still intact in the *wrky* mutant plants, alone, drives transcriptional induction of *Ipt*.

Plants recognize *A. tumefaciens* as a pathogen through pathogen-associated molecular patterns (PAMPs) and activate the innate immune responses and pathogen defense pathway, including mitogen-activated protein kinase (MAPK) cascade signaling [127,196,197]. The phosphorylation activities of at least three MAP kinases (MPKs) in *Arabidopsis*, MPK3, MPK4 and MPK6 are highly increased upon *A. tumefaciens* inoculation, and activation of the MAPKs is induced very fast, even within minutes [196]. MPK3 phosphorylates *Arabidopsis* VIP1 (virE2 interacting protein 1) to regulate *PR1* (pathogenesis-related genes 1) gene expression. The relocalization of the phosphorylated VIP1 into the plant nucleus is abused by *A. tumefaciens* for the transfer of the T-DNA complex into the nucleus [196]. In our study, the three WRKY transcription factor genes, *WRKY18*, *WRKY40* and *WRKY60* are induced within 72 hours upon *A. tumefaciens* inoculation (Figure 15). The *WRKY18* gene responds to wounding and was not significantly responsive to *A. tumefaciens* inoculation at early time points of infection (Figure 15). In contrast, transcription of *WRKY40* peaked at 2 hpi and that of *WRKY60* at 4 hpi specifically upon *A. tumefaciens* inoculation (Figure 15), suggesting that the latter two respond to *A. tumefaciens* infection. WRKY18, WRKY40 and WRKY60 are paralogous transcription factors which are classified as group IIa WRKYs according to a single Cys_2His_2 zinc-finger motif in their protein sequence. The three WRKYs cooperatively regulate biotic and abiotic stress responses in *Arabidopsis* [65,67,68,79,102,107,123]. The *WRKY18*, *WRKY40* and *WRKY60* genes are induced by bacterial and fungal pathogens [123,180], and also by salicylic acid (SA), abscisic acid (ABA), salt and drought stress [83,107]. It is reported that WRKY transcription factors act downstream of the MAPK cascade [122,127]. In *Arabidopsis*, WRKY25 and WRKY33 interact with MKS1 (MPK4 substrate 1) and WRKY33 is for example phosphorylated by MPK3/MPK6 [128,129]. WRKY40 is known to be a substrate for MPK1, MPK4, MPK5, MPK8 and MPK16, as was determined in a high-density protein microarrays phosphorylation assay [198]. In addition, WRKY40 has a stronger binding activity for a W-box sequence than WRKY18, whereas WRKY60 does not bind to W-box sequences *in vitro* [123]. This study shows that WRKY40 binds directly to the *Ipt* promoter *in vitro* and has also the strongest effect on *Ipt* promoter activation in plant cells (Figure 14A, 20).

4.4 *Agrobacterium tumefaciens* combines host pathogen defense and auxin signaling to regulate oncogene expression

In this study, it is shown that both the WRKY mediated pathogen defense pathway and auxin-dependent ARF signaling regulate the *Ipt* promoter in *Arabidopsis*. It has been proven that WRKY transcription factors interact with many kinds of proteins [122]. One of the important interactions is the WRKY-WRKY protein interaction. WRKY18, WRKY40 and WRKY60 interact with each other and themselves [123]. WRKY30 is found to interact with WRKY53, WRKY54, and WRKY70 in the yeast two hybrid assay [124]. WRKY13 interact with WRKY40, WRKY57 and WRKY13 itself [125]. In addition, it is also reported that WRKY transcription factors interact with MAPK, proteins containing a conserved FxxxVQxLTG or VQ motif, chromatin remodeling proteins, calmodulin, and many other kinds of proteins [122]. This study shows for the first time that ARF5 and WRKY18, WRKY40, WRKY60 interact with each other in the BiFC assay (Figure 18). Most ARFs contain four important domains, except of ARF3, ARF13 and ARF17, which lack domain III and IV and ARF23 which only has domain I [134]. Domain III and IV are localized at the C-terminus of ARFs and are important for dimerization and interaction with Aux/IAA proteins [132]. According to this study, the domain III and IV of ARF5 are also essential for the interaction with the three WRKY transcription factors (Figure 18D). The interaction of ARF5 with WRKY40, but not with WRKY18 and WRKY60 highly enhanced activation of the *Ipt* promoter and rank WRKY40 as most important transcriptional activator for *Ipt* gene expression (Figure 19).

The results of this study suggest the following model for the regulation of oncogene expression in plant host cells (Figure 26). *Agrobacterium tumefaciens* is recognized by plant cells and promote plant mitogen-activated protein kinase (MAPK) cascade signaling through pathogen-associated molecular patterns (PAMPs) within a few minutes [196,197]. When the MEKK1 (MAP kinase kinase kinase 1) - MKK1/MKK2 (MAP kinase kinase 1/2) - MPK4 (MAP kinase 4) cascade is activated MPK4 phosphorylates the WRKY40 transcription factor, of which the gene is early responsive to an *A. tumefaciens* infection. The activated WRKY40 binds to W-boxes within the *Ipt* promoter and promote *Ipt* expression. The bidirectional promoter for the *IaaH* and *IaaM* genes is recognized by the RNA polymerase II complex, which drives basal expression of *IaaH* and *IaaM* for auxin biosynthesis. In addition, auxin is secreted by *A. tumefaciens* into the apoplast. Expression of the *ARF5* gene is induced by auxin and controlled by ARF5 itself, which then binds to the AuxREs in the *Ipt* promoter. The Aux/IAA protein, IAA12, functions as repressor, which can inhibit activation of the *Ipt* promoter by ARF5. However, IAA12 proteins become unstable with increasing auxin levels and are degraded upon binding of auxin to the F-box protein TIR1. Domain II of IAA12 interacts with TIR1 which form the SCF^{TIR} ubiquitin ligase complex with Skp (ASK1) and Cullin (CUL1) for poly-ubiquitination of IAA12 [199,200]. Degradation of IAA12 releases the transcription factor ARF5, which then interacts via domain III and IV with WRKY40 to form a complex and boost expression of the *Ipt* oncogene.

From our study we conclude that *A. tumefaciens* utilizes both the WRKY40-dependend pathogen defense pathway and ARF5-mediated auxin signaling to regulate *Ipt* expression, which stimulates growth and differentiation. The *IaaH* and *IaaM* oncogenes are not regulated by plant transcription factors and expressed in a basal level.

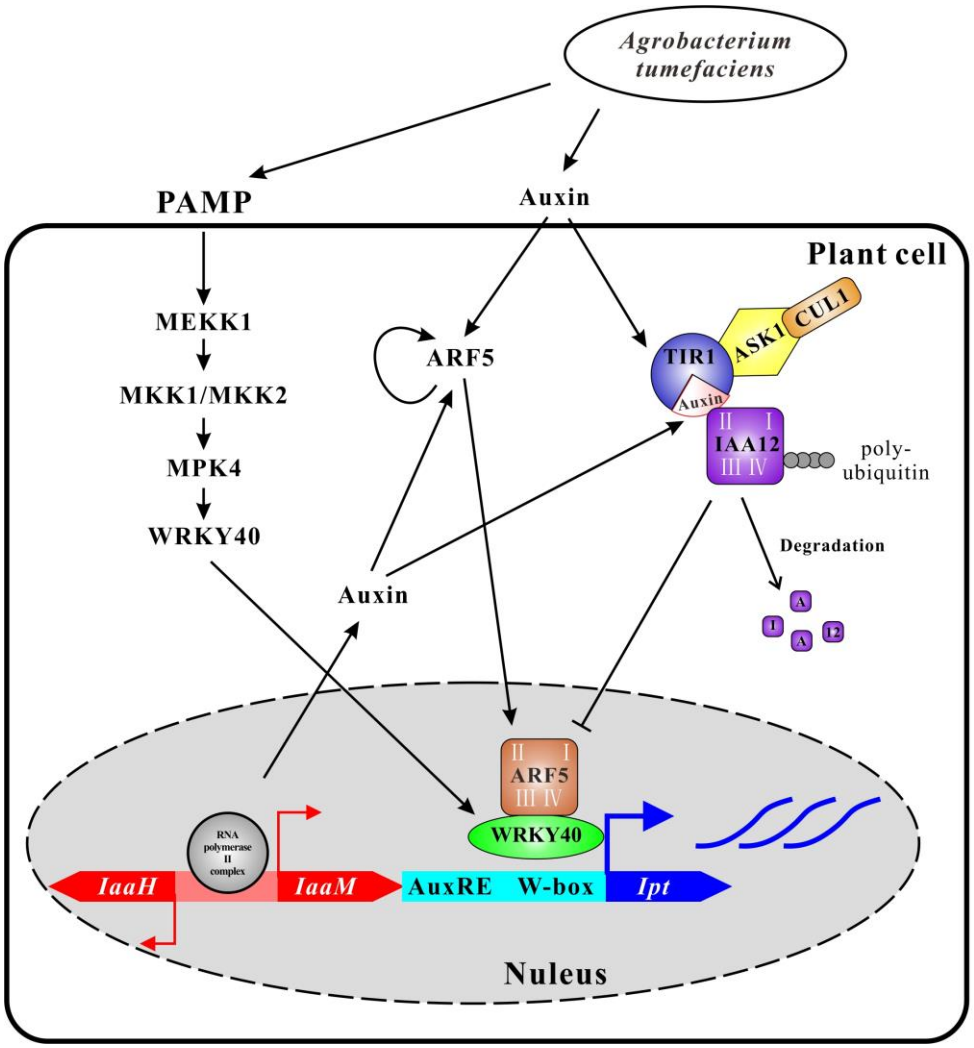


Figure 26. Proposed model of regulation of oncogene expression in the host cell.
Plants recognize *Agrobacterium tumefaciens* as a pathogen and activate PAMPs (pathogen-associated molecular patterns) induced MAPK cascades, MEKK1 (MAP kinase kinase kinase 1) -MKK1/MKK2 (MAP kinase kinase 1/2) - MPK4 (MAP kinase 4). The transcription factor WRKY40 is activated by MPK4-dependent phosphorylation and binds to W-boxes (TGAC) located in *Ipt* promoter to drive expression of the isopentenyl transferase (*Ipt)* gene. *IaaH* (indole-3-acetamide hydrolase) and *IaaM* (tryptophan monooxygenase) promoters are recognized by the RNA polymerase II complex and drive basal expression of *IaaH* and *IaaM* oncogenes. T-DNA transformed plant cells contain elevated auxin levels six days after inoculation due to the activity of the *IaaH* and *IaaM* enzymes and secretion by *A. tumefaciens* cells. Auxin induces *ARF5* expression and binds to the receptor TIR1 (transport inhibitor response 1) to recruit IAA12. TIR1 is a F-box protein, which forms a complex with SKP (ASK1) and cullin1 (CUL1). This SCF (SKP, cullin1, F-box protein) E3 ubiquitin ligase complex catalyzes polyubiquitination of IAA12. The degradation of IAA12 by the proteasome releases ARF5 which controls its own gene expression (circular arrow). ARF5 binds to the AuxREs (TGTCNC or TGTCTN) in the *Ipt* promoter and forms a complex with WRKY40 via domain III and IV. The ARF5-WRKY40 complex finally enhances activation of the *Ipt* promoter and promotes expression of the *Ipt* oncogene.

5. References

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6. Appendix

6.1 Supplement Table

Table S1 List of transcription factor genes differentially regulated upon infection with *Agrobacterium tumefaciens* strain C58. Analyses are based on microarray data [28,174] using annotation of the Plant Transcription Factor Database v3.0 [58] (http://planttfdb.cbi.pku.edu.cn/index.php?sp=Ath). Fold change (FCh) ≥ 2 or ≤ 0.5, p value < 0.01; hpi: hours postinoculation; dpi: days postinoculation.

Locus	FCh	P Value	Family	Description	PTA
3 hpi					
AT4G23810	2.47	0.000	WRKY	WRKY53 (WRKY DNA-binding protein 53)	In
AT1G80840	2.22	0.001	WRKY	WRKY40 (WRKY DNA-binding protein 40)	In
AT5G63790	2.18	0.001	NAC	ANAC102 (Arabidopsis NAC domain containing protein 102)	
6 dpi					
AT5G12330	2.76	0.000	SRS	LRP1 (LATERAL ROOT PRIMORDIUM 1)	In
AT5G65640	2.36	0.000	bHLH	BHLH093 (BETA HLH PROTEIN 93)	
AT4G17980	2.24	0.000	NAC	ANAC071 (Arabidopsis NAC domain containing protein 71)	In
AT5G25190	2.23	0.000	ERF	ethylene-responsive element-binding protein, putative	In
AT2G47260	2.22	0.000	WRKY	WRKY23 (WRKY DNA-binding protein 23)	In
AT1G10585	2.15	0.000	bHLH	transcription factor	
AT5G57660	0.49	0.000	CO-like	zinc finger (B-box type) family protein	
AT5G61600	0.48	0.000	ERF	ethylene-responsive element-binding family protein	In
AT1G74840	0.48	0.000	MYB related	myb family transcription factor	In
AT2G28200	0.47	0.000	C_2H_2	nucleic acid binding / transcription factor/ zinc ion binding	
AT5G49450	0.46	0.000	GRAS	ATBZIP1 (ARABIDOPSIS THALIANA BASIC LEUCINE-ZIPPER 1)	
AT5G61590	0.35	0.000	ERF	AP2 domain-containing transcription factor family protein	In
35 dpi tumor					
AT2G47260	11.63	0.000	WRKY	WRKY23 (WRKY DNA-binding protein 23)	In
AT1G31320	9.85	0.000	LBD	LBD4 (LOB DOMAIN-CONTAINING PROTEIN 4)	
AT3G02550	9.63	0.000	LBD	LBD41 (LOB DOMAIN-CONTAINING PROTEIN 41)	
AT5G60200	9.30	0.000	Dof	Dof-type zinc finger domain-containing protein	In

AT5G25190	8.72	0.000	ERF	ethylene-responsive element-binding protein, putative	In
AT5G12330	7.83	0.000	SRS	LRP1 (LATERAL ROOT PRIMORDIUM 1)	In
AT2G31180	5.64	0.000	MYB	AtMYB14/Myb14at (myb domain protein 14)	
AT1G62300	5.52	0.001	WRKY	WRKY6 (WRKY DNA-binding protein 6)	In
AT1G01010	5.06	0.000	NAC	ANAC001 (Arabidopsis NAC domain containing protein 1)	In
AT1G19850	4.93	0.000	ARF	MP (MONOPTEROS)	In
AT3G10040	4.81	0.000	Trihelix	transcription factor	
AT5G25160	4.80	0.000	C_2H_2	ZFP3 (ZINC FINGER PROTEIN 3)	
AT1G05710	4.61	0.000	bHLH	ethylene-responsive protein, putative	In
AT3G25730	4.26	0.000	RAV	AP2 domain-containing transcription factor, putative	In
AT2G34710	4.15	0.000	HD-ZIP	PHB (PHABULOSA)	
AT5G13330	4.10	0.000	ERF	RAP2.6L (related to AP2 6L)	In
AT5G47370	4.09	0.007	HD-ZIP	HAT2	
AT1G68320	4.06	0.000	MYB	MYB62 (myb domain protein 62)	
AT1G36060	4.05	0.000	ERF	AP2 domain-containing transcription factor, putative	
AT1G06180	4.00	0.000	MYB	ATMYB13 (myb domain protein 13)	In
AT5G13080	3.79	0.002	WRKY	WRKY75 (WRKY DNA-BINDING PROTEIN 75)	
AT2G36080	3.71	0.001	B3	DNA-binding protein, putative	In
AT2G01430	3.53	0.007	HD-ZIP	homeobox-leucine zipper protein 17 (HB-17) / HD-ZIP transcription factor 17	
AT3G04670	3.51	0.001	WRKY	WRKY39 (WRKY DNA-binding protein 39)	
AT5G61890	3.51	0.000	ERF	AP2 domain-containing transcription factor family protein	
AT5G10510	3.39	0.000	AP2	AIL6 (AINTEGUMENTA-LIKE 6)	In
AT3G03660	3.32	0.009	WOX	DNA binding / transcription factor	
AT3G01970	3.25	0.001	WRKY	WRKY45 (WRKY DNA-binding protein 45)	
AT3G23240	3.23	0.004	ERF	ATERF1/ERF1 (ETHYLENE RESPONSE FACTOR 1)	In
AT3G61850	3.11	0.001	Dof	DAG1 (DOF AFFECTING GERMINATION 1)	In
AT4G37750	3.10	0.001	AP2	ANT (AINTEGUMENTA)	In
AT1G29160	3.07	0.005	Dof	Dof-type zinc finger domain-containing protein	In
AT5G03680	3.05	0.001	Trihelix	PTL (PETAL LOSS)	In
AT5G22570	2.97	0.006	WRKY	WRKY38 (WRKY DNA-binding protein 38)	
AT5G66700	2.94	0.000	HD-ZIP	HB53 (homeobox-8)	In
AT1G57560	2.90	0.000	MYB	AtMYB50 (myb domain protein 50)	In
AT5G65510	2.88	0.000	AP2	AIL7 (AINTEGUMENTA-LIKE 7)	
AT3G24120	2.88	0.000	G2-like	myb family transcription factor	
AT4G32880	2.85	0.002	HD-ZIP	ATHB-8 (HOMEOBOX GENE 8)	In

AT2G47520	2.82	0.000	ERF	AP2 domain-containing transcription factor, putative	
AT5G39610	2.76	0.004	NAC	ANAC092/ATNAC2/ATNAC6 (Arabidopsis NAC domain containing protein 92)	In
AT2G18550	2.71	0.008	HD-ZIP	ATHB21/HB-2 (homeobox-2)	
AT5G49620	2.71	0.007	MYB	AtMYB78 (myb domain protein 78)	
AT1G80730	2.68	0.003	C_2H_2	ZFP1 (ARABIDOPSIS THALIANA ZINC-FINGER PROTEIN 1)	
AT5G17490	2.57	0.000	GRAS	RGL3 (RGA-LIKE 3)	
AT2G41690	2.52	0.001	HSF	AT-HSFB3 (Arabidopsis thaliana heat shock transcription factor B3)	
AT5G57390	2.52	0.000	AP2	AIL5 (AINTEGUMENTA-LIKE 5)	
AT5G49450	2.50	0.005	GRAS	ATBZIP1 (ARABIDOPSIS THALIANA BASIC LEUCINE-ZIPPER 1)	
AT5G17300	2.47	0.002	MYB related	myb family transcription factor	In
AT1G02220	2.46	0.004	NAC	ANAC003 (Arabidopsis NAC domain containing protein 3)	In
AT4G29190	2.45	0.001	C_3H	zinc finger (CCCH-type) family protein	
AT2G23320	2.44	0.000	WRKY	WRKY15 (WRKY DNA-binding protein 15)	In
AT4G37540	2.42	0.007	LBD	LBD39 (LOB DOMAIN-CONTAINING PROTEIN 39)	
AT5G15160	2.40	0.004	bHLH	bHLH family protein	In
AT3G45610	2.38	0.000	Dof	Dof-type zinc finger domain-containing protein	In
AT5G03790	2.34	0.000	HD-ZIP	ATHB51/LMI1 (LATE MERISTEM IDENTITY1)	
AT1G46264	2.31	0.002	HSF	AT-HSFB4 (Arabidopsis thaliana heat shock transcription factor B4)	
AT2G43000	2.28	0.001	NAC	ANAC042 (Arabidopsis NAC domain containing protein 42)	
AT2G34140	2.22	0.001	Dof	Dof-type zinc finger domain-containing protein	In
AT1G80590	2.18	0.000	WRKY	WRKY66 (WRKY DNA-binding protein 66)	
AT2G46590	2.18	0.003	Dof	DAG2 (DOF AFFECTING GERMINATION 2)	
AT4G37650	2.16	0.001	GRAS	SHR (SHORT ROOT)	
AT1G79180	2.15	0.000	MYB	AtMYB63 (myb domain protein 63)	
AT4G17980	2.15	0.002	NAC	ANAC071 (Arabidopsis NAC domain containing protein 71)	In
AT1G69780	2.14	0.000	HD-ZIP	ATHB13	In
AT5G46590	2.12	0.002	NAC	ANAC096 (Arabidopsis NAC domain containing protein 96)	
AT4G36160	2.08	0.000	NAC	ANAC076/VND2 (VASCULAR-RELATED NAC-DOMAIN 2)	
AT5G39660	2.06	0.008	Dof	CDF2 (CYCLING DOF FACTOR 2)	

AT2G37590	2.05	0.007	Dof	Dof-type zinc finger domain-containing protein	In
AT5G13910	2.05	0.006	ERF	LEP (LEAFY PETIOLE)	In
AT1G68840	2.05	0.001	RAV	RAV2 (REGULATOR OF THE ATPASE OF THE VACUOLAR MEMBRANE)	In
AT5G65790	2.05	0.001	MYB	MYB68 (myb domain protein 68)	
AT1G30490	2.03	0.001	HD-ZIP	PHV (PHAVOLUTA)	
AT1G69690	0.50	0.006	TCP	TCP family transcription factor, putative	In
AT5G07580	0.50	0.007	ERF	DNA binding / transcription factor	In
AT5G15850	0.50	0.006	CO-like	COL1 (CONSTANS-LIKE 1)	
AT1G76890	0.50	0.003	Trihelix	GT2	
AT2G35550	0.49	0.000	BBR-BPC	ATBPC7/BBR/BPC7/BPC7 (BASIC PENTACYSTEINE 7)	
AT4G17460	0.49	0.001	HD-ZIP	HAT1 (homeobox-leucine zipper protein 1)	
AT2G14210	0.49	0.008	MIKC	ANR1	
AT3G14020	0.48	0.000	NF-YA	CCAAT-binding transcription factor (CBF-B/NF-YA) family protein	
AT3G28920	0.48	0.000	ZF-HD	ATHB34 (ARABIDOPSIS THALIANA HOMEOBOX PROTEIN 34)	
AT3G59580	0.48	0.000	Nin-like	RWP-RK domain-containing protein	
AT1G27360	0.47	0.000	SBP	squamosa promoter-binding protein-like 11 (SPL11)	
AT3G11020	0.47	0.000	ERF	DREB2B (DRE-binding protein 2B)	In
AT3G61890	0.46	0.006	HD-ZIP	ATHB-12 (ARABIDOPSIS THALIANA HOMEOBOX PROTEIN 12)	In
AT4G00050	0.46	0.004	bHLH	UNE10 (unfertilized embryo sac 10)	
AT1G56170	0.46	0.007	NF-YC	HAP5B (Heme activator protein (yeast) homolog 5B)	
AT2G43010	0.46	0.001	bHLH	PIF4 (PHYTOCHROME INTERACTING FACTOR 4)	
AT1G30500	0.45	0.001	NF-YA	CCAAT-binding transcription factor (CBF-B/NF-YA) family protein	
AT5G47640	0.45	0.004	NF-YB	CCAAT-box binding transcription factor subunit B (NF-YB) (HAP3) (AHAP3) family (Hap3b)	
AT4G36930	0.44	0.001	bHLH	SPT (SPATULA)	
AT1G70510	0.44	0.001	TALE	KNAT2 (KNOTTED-LIKE FROM ARABIDOPSIS THALIANA 2)	
AT2G33480	0.44	0.007	NAC	ANAC041 (Arabidopsis NAC domain containing protein 41)	
AT5G56860	0.43	0.000	GATA	GNC (GATA, NITRATE-INDUCIBLE, CARBON METABOLISM-INVOLVED)	
AT3G21270	0.43	0.003	Dof	ADOF2 (Arabidopsis dof zinc finger protein 2)	In
AT1G22070	0.42	0.000	bZIP	TGA3 (TGA1a-related gene 3)	In
AT4G17880	0.42	0.002	bHLH	basic helix-loop-helix (bHLH) family protein	

AT2G27220	0.41	0.003	TALE	BLH5 (BELL1-LIKE HOMEODOMAIN 5)	
AT1G54160	0.41	0.000	NF-YA	CCAAT-binding transcription factor (CBF-B/NF-YA) family protein	
AT5G18240	0.41	0.001	G2-like	MYR1 (MYB-RELATED PROTEIN 1)	
AT2G02070	0.41	0.000	C_2H_2	ATIDD5 (ARABIDOPSIS THALIANA INDETERMINATE(ID)-DOMAIN 5)	
AT4G08150	0.40	0.004	TALE	KNAT1 (BREVIPEDICELLUS 1)	
AT1G26960	0.40	0.002	HD-ZIP	ATHB23 (ARABIDOPSIS THALIANA HOMEOBOX PROTEIN 23)	In
AT2G01570	0.40	0.000	GRAS	RGA1 (REPRESSOR OF GA1-3 1)	
AT2G20570	0.38	0.000	G2-like	GPRI1 (GOLDEN2-LIKE 1)	In
AT5G28300	0.35	0.000	Trihelix	trihelix DNA-binding protein, putative	In
AT4G00730	0.35	0.001	HD-ZIP	ANL2 (ANTHOCYANINLESS 2)	
AT2G35940	0.34	0.001	TALE	BLH1 (embryo sac development arrest 29)	
AT3G11090	0.34	0.000	LBD	LBD21 (LOB DOMAIN-CONTAINING PROTEIN 21)	
AT1G61660	0.34	0.000	bHLH	basic helix-loop-helix (bHLH) family protein	In
AT1G74660	0.33	0.000	ZF-HD	MIF1 (MINI ZINC FINGER 1)	
AT2G02080	0.32	0.000	C_2H_2	ATIDD4 (ARABIDOPSIS THALIANA INDETERMINATE(ID)-DOMAIN 4)	
AT5G60850	0.32	0.000	Dof	OBP4 (OBF BINDING PROTEIN 4)	In
AT2G33810	0.32	0.000	SBP	SPL3 (SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 3)	
AT4G36870	0.31	0.000	TALE	BLH2 (BEL1-LIKE HOMEODOMAIN 2, SAWTOOTH 1)	
AT1G71030	0.30	0.001	MYB related	ATMYBL2 (Arabidopsis myb-like 2)	
AT2G47890	0.30	0.001	DBB	zinc finger (B-box type) family protein	
AT5G61420	0.30	0.001	MYB	MYB28 (MYB DOMAIN PROTEIN 28)	
AT1G52880	0.29	0.001	NAC	NAM (Arabidopsis NAC domain containing protein 18)	
AT5G44190	0.28	0.000	G2-like	GLK2 (GOLDEN2-LIKE 2)	In
AT5G62000	0.27	0.006	ARF	ARF2 (AUXIN RESPONSE FACTOR 2)	
AT2G38090	0.26	0.002	MYB	myb family transcription factor	
AT2G03710	0.26	0.000	MIKC	SEP4 (SEPALLATA4)	In
AT2G44745	0.26	0.007	WRKY	WRKY family transcription factor	In
AT1G18710	0.25	0.005	MYB	AtMYB47 (myb domain protein 47)	
AT3G59060	0.25	0.003	bHLH	PIL6 (PHYTOCHROME-INTERACTING FACTOR 5)	
AT1G55110	0.24	0.000	C_2H_2	ATIDD7 (ARABIDOPSIS THALIANA INDETERMINATE(ID)-DOMAIN 7)	
AT1G32640	0.24	0.004	bHLH	ATMYC2 (JASMONATE INSENSITIVE 1)	
AT4G28530	0.24	0.000	NAC	ANAC074 (Arabidopsis NAC domain containing protein 74)	
AT5G60910	0.22	0.001	MIKC	AGL8 (AGAMOUS-LIKE 8)	

AT3G05690	0.21	0.000	NF-YA	ATHAP2B/HAP2B/UNE8 (HEME ACTIVATOR PROTEIN (YEAST) HOMOLOG 2B)	
AT3G53310	0.20	0.000	B3	transcriptional factor B3 family protein	In
AT5G06510	0.20	0.000	NF-YA	CCAAT-binding transcription factor (CBF-B/NF-YA) family protein	
AT2G17040	0.19	0.000	NAC	ANAC036 (Arabidopsis NAC domain containing protein 36)	
AT1G66390	0.19	0.006	MYB	PAP2 (PRODUCTION OF ANTHOCYANIN PIGMENT 2)	
AT1G56650	0.19	0.002	MYB	PAP1 (PRODUCTION OF ANTHOCYANIN PIGMENT 1)	
AT5G59780	0.16	0.001	MYB	MYB59 (myb domain protein 59)	
AT1G62360	0.14	0.001	TALE	STM (SHOOT MERISTEMLESS)	
AT3G46130	0.13	0.000	MYB	MYB111 (myb domain protein 111)	

Table S2 List of primer used for cloning.

Primer Name	Sequence	Length of PCR product	Description
Luc user fwd	GGCTTAAU**TAAGGATCCTTAATTAAACCTCAGC**ATGGAAGACGCTAAAAACATAA	1902 bp	Luciferase reporter gene and CAMV terminator into pSAT-2221 for luc+/pSAT-2221 with a new new PacI cassette
CAMV Term user rev	GGTTTAAUATCGATCTGGATTTTAGTACTGG		
35S USER fwd	GGCTTAAUTCTAGAGATCCGTCAACATGGTGG	759 bp	35s promoter into luc+/pSAT-2221 for 35s luc+/pSAT-2221
35S USER rev	GGTTTAAUTCCTCTCCAAATGAAATGAACTTCC		
35S mini User fwd	GGCTTAAU**TAAGGATCCTTAATTAAACCTCAGC**GCAAGACCCTTCCTCTATATAAG	47 bp (with 35S USER rev)	35s minimol promoter into luc+/pSAT-2221for 35s mini luc+/pSAT-2221 witha new new PacI cassette
IaaH pro USER fwd	GGCTTAAUTGCTAGAAAAGATTGGTCTTTGTG	337 bp	*IaaH* promoter into luc+/pSAT-2221 for IaaH pro luc+/pSAT-2221
IaaH pro USER rev	GGTTTAAUTTTTCTGGTTTGGGGATTTCAG		
IaaM pro USER fwd	GGCTTAAUTTTTCTGGTTTGGGGATTTCAG	337 bp	*IaaM* promoter into Luc+/pSAT-2221 for IaaM pro luc+/pSAT-2221
IaaM pro User rev	GGTTTAAUTGCTAGAAAAGATTGGTCTTTGTG		
Ipt pro USER fwd	GGCTTAAUTCTACGGATCCTGTTACAAGTATT	697 bp	*Ipt* promoter into luc+/pSAT-2221 for Ipt pro luc+/pSAT-2221
Ipt pro USER rev	GGTTTAAUAAGTTTTTTGCGGTATCTTGAATAC		
Ipt e User fwd	GGCTTAAUTCGTTAGTGACAAATTGCTTTC	60 bp	*Ipt* promoter element into luc+/pSAT-2221 for Ipt e pro luc+/pSAT-2221
Ipt e User rev	GGTTTAAUTTGTTTTTCAACAAAGTGTGG		

IaaH SacI fwd	GCGAGCTCTGCTAGAAAAGATTGGTCTTTGTG	337 bp	*IaaH* promoter into pHISi for IaaH pro/pHISi
IaaH Xbal rev	GCTCTAGATTTTCTGGTTTGGGGATTTCAG		
IaaM SacI fwd	GCGAGCTCTTTTCTGGTTTGGGGATTTCAG	337 bp	*IaaM* promoter into pHISi for IaaM pro/pHISi
IaaM Xbal rev	GCTCTAGATGCTAGAAAAGATTGGTCTTTGTG		
Ipt EcoRI fwd	CGGAATTCTCTACGGATCCTGTTACAAGTATT	697 bp	*Ipt* promoter into pHISi for Ipt pro/pHISi
Ipt Xbal rev	GCTCTAGAAAGTTTTTTGCGGTATCTTGAATAC		
Ipt pro 100 rev XbaI	GCTCTAGACAACTGGCTTTACCGTCTCC	597 bp (with Ipt EcoRI fwd)	Ipt promoter without TATA box region into pHISi for Ipt pro ΔTATA/pHISi
Ipt pro element fwd EcoRI	AATTCTCGTTAGTGACAAATTGCTTTCAAGGAGACAGCCATGCCCCACACTTTGTTGAAAAACAAT	60 bp	Ipt promoter element into pHISi for Ipt elment/pHISi
Ipt pro element rev Xba I	CTAGATTGTTTTTCAACAAAGTGTGGGGCATGGCTGTCTCCTTGAAAGCAATTTGTCACTAACGAG		
HIS gene fwd XbaI	GCTCTAGAATGACAGAGCAGAAAGCCC	624 bp	For Δpmini/pHISi
HIS gene rev KpnI	GGGGTACCGGTACCATTGGGCGAGGT		
ARF5 USER fwd	GGCTTAAUATGATGGCTTCATTGTCTTGT	2706 bp	CDS of ARF5 without stop code into pSAT #18 or pSAT #20 for ARF5/pSAT #18 or ARF5/pSAT #20
ARF5 USER CC rev	GGTTTAAUCCTGAAACAGAAGTCTTAAGATCG		
WRKY40 USER fwd	GGCTTAAUATGGATCAGTACTCATCCTCTTTG	906 bp	CDS of WRKY40 without stop code into pSAT #18 or pSAT #20 for WRKY40/pSAT #18 or WRKY40/pSAT #20
WRKY40 USER CC rev	GGTTTAAUCCTTTCTCGGTATGATTCTGTTGATA		
WRKY18 USER fwd	GGCTTAAUATGGACGGTTCTTCGTTTCTC	930 bp	CDS of WRKY18 without stop code into pSAT #18 or pSAT #20 for WRKY18/pSAT #18 or WRKY18/pSAT #20
WRKY18 USER CC rev	GGTTTAAUCCTGTTCTAGATTGCTCCATTAACC		
WRKY60 USER fwd	GGCTTAAUATGGACTATGATCCCAACACC	813 bp	CDS of WRKY60 without stop code into pSAT #18 or pSAT #20 for WRKY60/pSAT #18 or WRKY60/pSAT #20
WRKY60 USER CC rev	GGTTTAAUCCTGTTCTTGAATGCTCTATCAATCT		
ARF3 USER fwd	GGCTTAAUATGGGTGGTTTAATCGATCTGAA	1824 bp	CDS of ARF3 without stop code into pSAT #18 or pSAT #20 for ARF3/pSAT #18 or ARF3/pSAT #20
ARF3 USER CC rev	GGTTTAAUCCGAGAGCAATGTCTAGCAACATG		
ARF5 722 USER CC rev	GGTTTAAUCCCAAACAACCAGAAGGGTGGTTC	2166 bp (with ARF5 USER fwd)	1 722 aa of ARF5 into pSAT #18 or pSAT #20 for ARF5 Δ722/pSAT #18 or ARF5 Δ722/pSAT #20

WRKY53 USER fwd	GGCTTAAUATGGAAGGAAGAGATATGTTAAGTTG	972 bp	CDS of WRKY53 without stop code into pSAT #18 or pSAT #20 for WRKY53/pSAT #18 or WRKY53/pSAT #20
WRKY53 USER CC rev	GGTTTAAUCCATAATAAATCGACTCGTGTAAAAACGC		
WRKY40 fwd NdeI	GGGTTTCATATGGATCAGTACTCATCCTCTTTG	909 bp	CDS of WRKY40 with stop code into pET28b for WRKY40/pET28b
WRKY40 rev XhoI	CCGCTCGAGCTATTTCTCGGTATGATTCTGTTG		

Table S2 List of primer used for site mutation

Primer Name	Sequence	Description
AuxRE 1m fwd	TTAAAATATTAACTG**G**CGCATTTATTGAAAT	Site mutation for AuxRE 1 within *Ipt* promoter
AuxRE 1m rev	ATTTCAATAAATGCG**C**CAGTTAATATTTTAA	
AuxRE 2m fwd	AAAGATCTCACTCTG**G**CGCCAGCAATGGTGT	Site mutation for AuxRE 2 within *Ipt* promoter
AuxRE 2m rev	ACACCATTGCTGGCG**C**CAGAGTGAGATCTTT	
AuxRE 3m fwd	GTGTAATCAGCGCAG**C**CAAGTGGCAGTAAAG	Site mutation for AuxRE 3 within *Ipt* promoter
AuxRE 3m rev	CTTTACTGCCACTTG**G**CTGCGCTGATTACAC	
AuxRE 4m fwd	GTGCCCTCGTTAGTG**C**CAAATTGCTTTCAAG	Site mutation for AuxRE 4 within *Ipt* promoter
AuxRE 4m rev	CTTGAAAGCAATTTG**G**CACTAACGAGGGCAC	
AuxRE 5m fwd	ATTGCTTTCAAGGAG**C**CAGCCATGCCCCAC	Site mutation for AuxRE 5 within *Ipt* promoter
AuxRE 5m Rev	GTGGGGCATGGCTG**G**CTCCTTGAAAGCAAT	

6.2 Supplement Figure

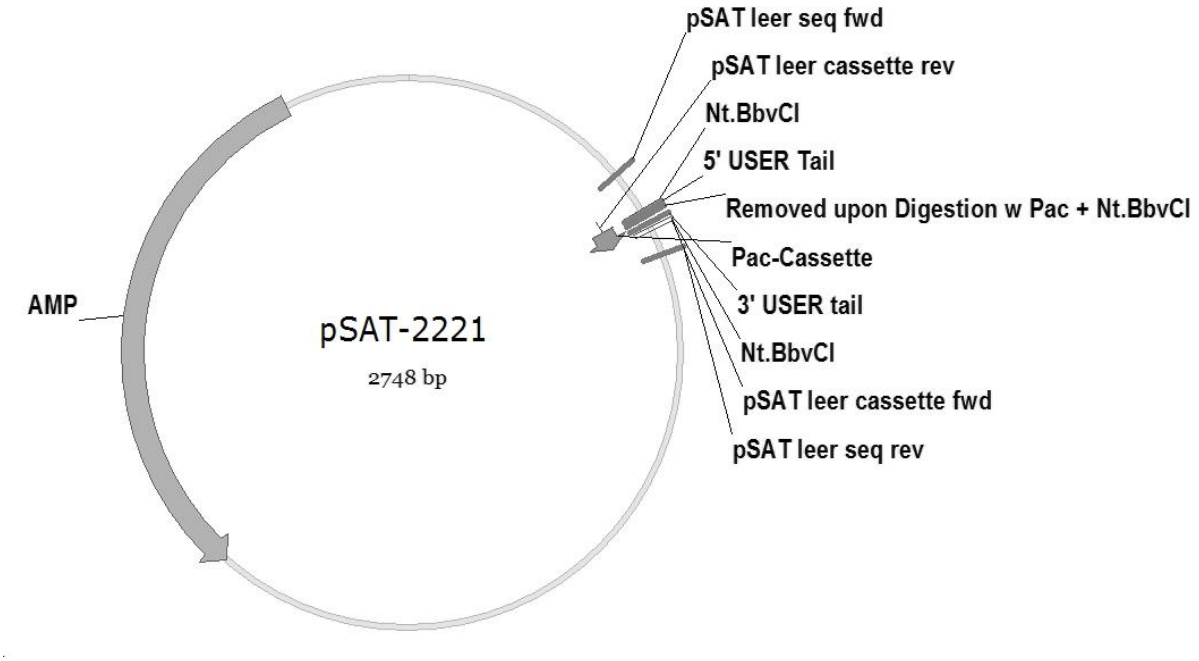

Figure S1. The map of vector pSAT-2221 (2.12).
The vector pSAT-2221 is USER vector and used for generation of luc+/pSAT-2221 reporter vector. DNA fragments of luciferase reporter gene and CAMV-terminator were amplified by Phusion Cx polymerase using USER primers and inserted into pSAT by USER cloning (2.8.3). A new PacI cassette is introduced 5' upstream of luciferase reporter gene for next USER cloning.

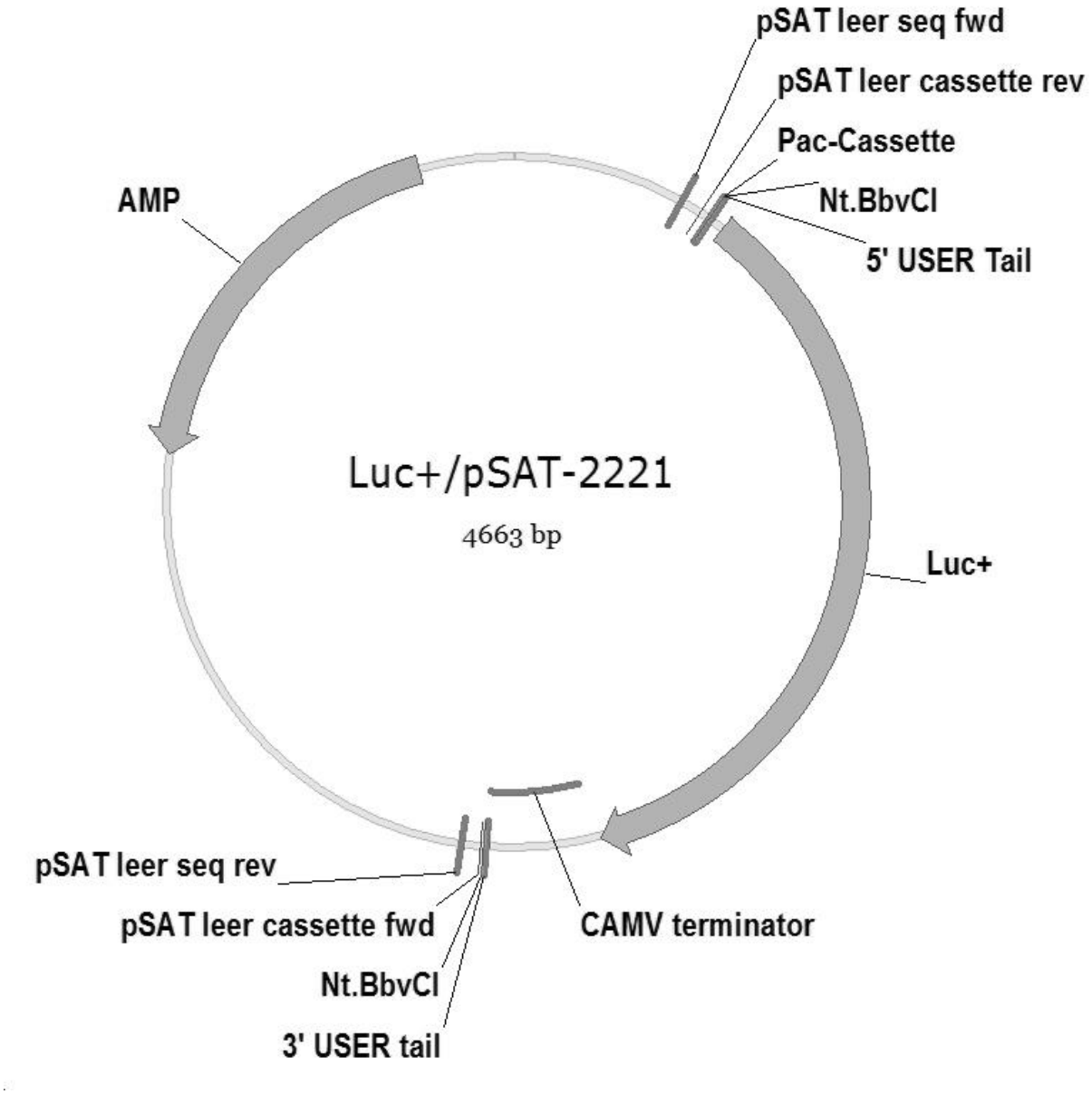

Figure S2. The map of vector luc+/pSAT-2221 (2.12).
The vector luc+/pSAT-2221 is USER vector and used for generation of *IaaH* pro luc+/pSAT-2221, *IaaM* pro luc+/pSAT-2221 and *Ipt* pro luc+/pSAT-2221 reporter plasmid. The promoters and 5' untraslated regrions (5' UTR) of *IaaH*, *IaaM* and *Ipt* were amplified by Phusion Cx polymerase using USER primers and inserted into luc+/pSAT by USER cloning (2.8.3).

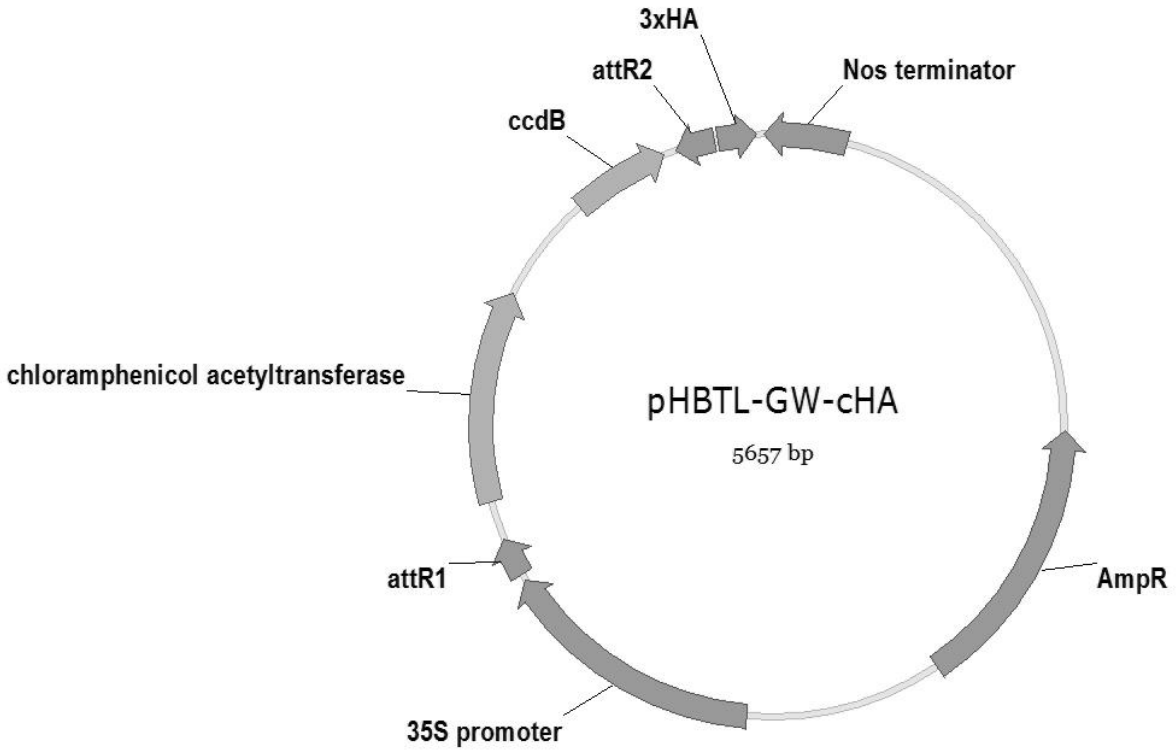

Figure S3. The map of vector pHBTL (2.12).
The vector pHBTL is GATEWAY destination vector. The transcription factor expression plasmid of PTA system was generated by LR reaction of entry vector and pHBTL destination vector. All of the transcription factor expression plasmids of PTA system were kindly provided by Prof. Wolfgang Dröge-Laser (Julius-von-Sachs-Institute, Pharmaceutical Biology, University of Wuerzburg, Wuerzburg, Germany) [156].

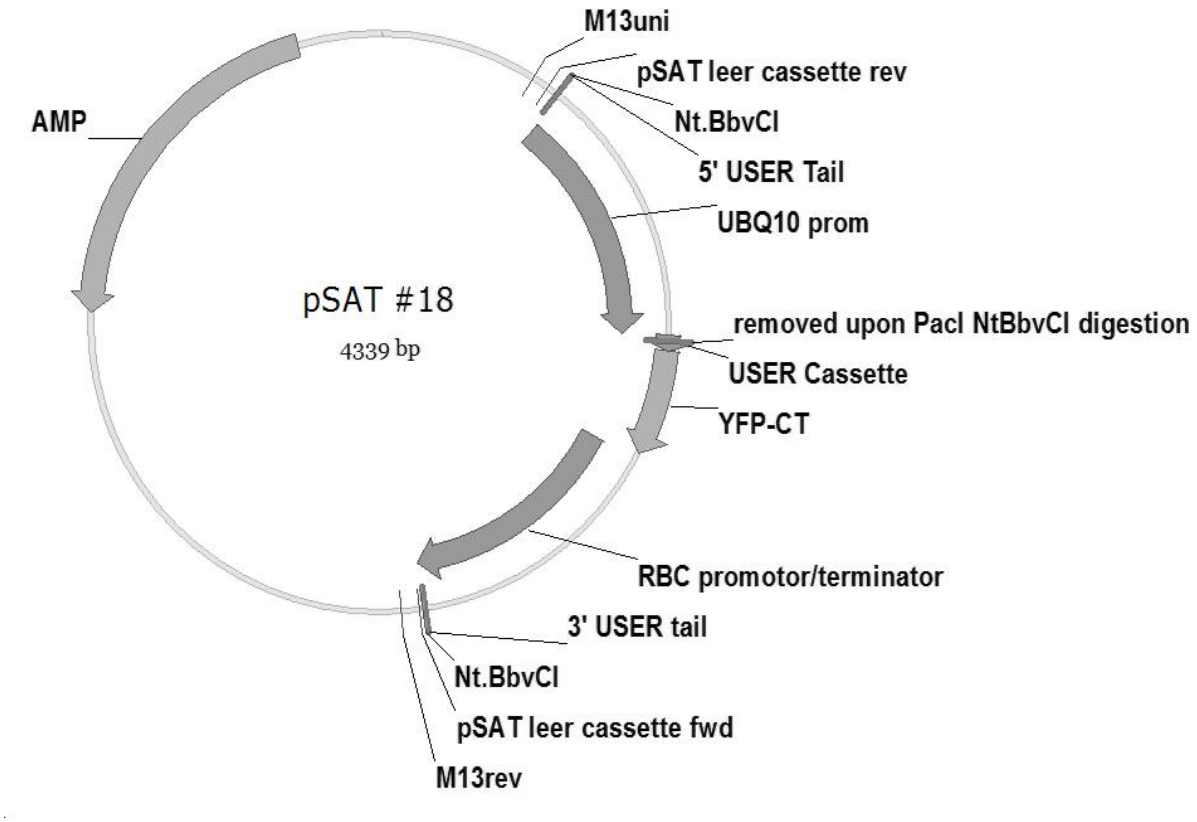

Figure S4. The map of vector pSAT #18 (2.13).
The vector pSAT #18 is USER vector for cYFP fusion proteins for BiFC experiments. The full length of ARF or WRKY CDS was amplified by Phusion Cx polymerase using USER primers and inserted into pSAT #18 by USER cloning (2.8.3).

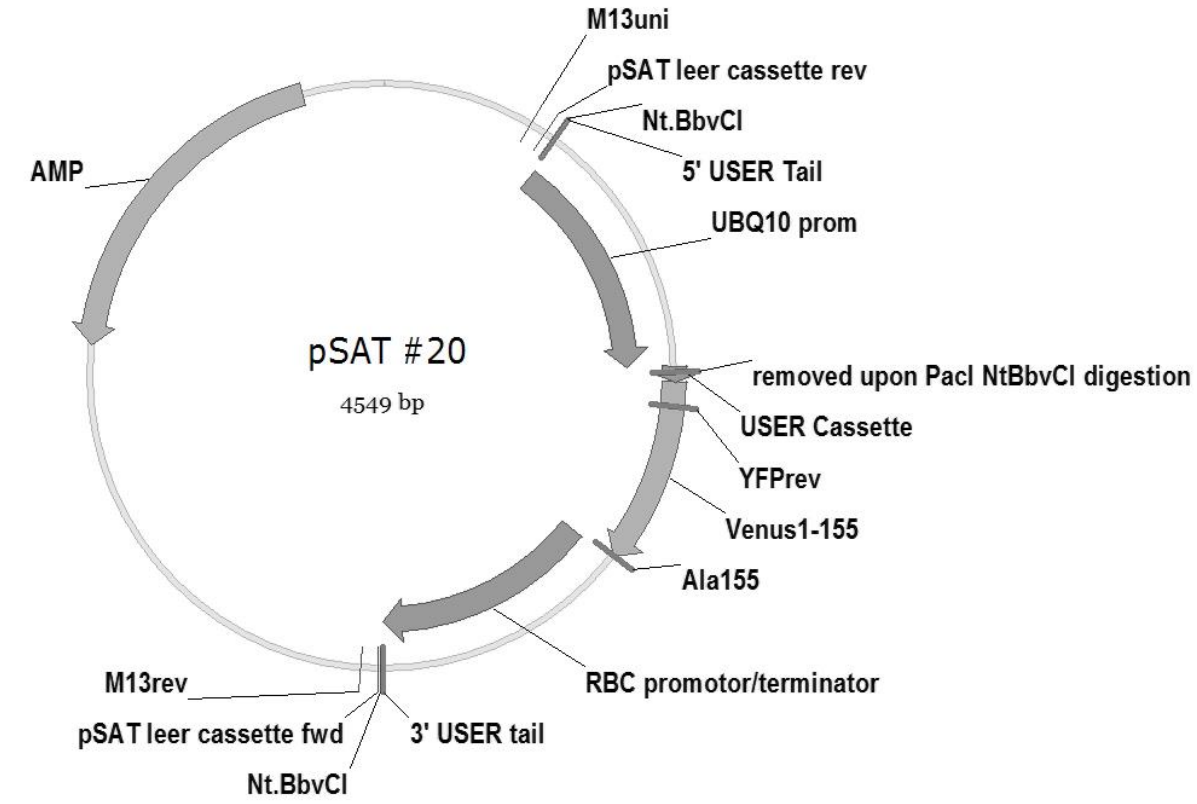

Figure S5. The map of vector pSAT #20 (2.13).
The vector pSAT #18 is USER vector for nYFP fusion proteins for BiFC experiments. The full length of ARF or WRKY CDS was amplified by Phusion Cx polymerase using USER primers and inserted into pSAT #20 by USER cloning (2.8.3).

6.3 Abbreviation

1-NAA	1-Naphthaleneacetic acid
2,4-D	2,4-Dichlorophenoxyacetic acid
3-AT	3-amino-1,2,4-triazole
4-Cl-IAA	4-chloroindole-3-acetic acid
5’ RACE	5’- rapid amplification of cDNA ends
5’ UTR	5’ untraslated regrions
6-BA	6-benzylaminopurine
ABA	abscisic acid
AD	activation domain
AFB	auxin signaling F box
AMP	adenosine monophosphate
APS	ammonium persulfate
ARF	auxin response factor
At	*Arabidopsis thaliana*
ATP	adenosine 5'-triphosphate
AtSkp1	*A. thaliana* S-phase kinase-associated protein 1 homolog
Aux/IAA	auxin/indole acetic acid
AuxRE	auxin response elements
BiFC	Bimolecular Fluorescence Complementation
bp	base pair
BSA	bovine serum albumin
bZIP	basic leucine-zipper
CBF	*cyt-1* binding factor
cDNA	complementary DNA
CDS	coding sequence
CPPU	*N*-phenyl-*N’*-(2-chloro-4-pyridyl) urea
Ct	threshold cycle
cYFP	C-terminal half of YFP
cZ	*cis*-zeatin
DBD	DNA binding domain
DMAPP	dimethylallyl diphosphate (or pyrophosphate)
DNA	desoxyribonucleic acid
dNTP	desoxynucleoside triphosphate
dpi	days postinoculation
DPU	*N,N’*-phenylurea
dpw	post wounding
DTT	dithiothreitol
dTTP	desoxythymidylate triphosphate
DZ	dihydrozeatin
EDTA	ethylenediaminetetraaceticacid
EMSA	Electrophoretic Mobility Shift Assay
ESTs	expressed sequence tags
ET	ethylene
fg	femtogram

g	gram
GFP	green fluorescent protein
GUS	β-glucuronidase
h	hour
HD	homeodomain
HLH	helix-loop-helix
HMBDP	1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate
hpi	hours postinoculation
HPLC	high performance liquid chromatography
HR	hypersensitive response
HTAM	N-hydroxy-TAM
IAA	indole-3-acetic acid
IAA-Asp	IAA-aspartate
IaaH	indole-3-acetamide hydrolase
IAAld	indole-3-acetaldehyde
IaaM	tryptophan monooxygenase
IAM	indole-3-acetamide
IAN	indole-3-acetonitrile
IAOx	indole-3-acetaldoxime
IBA	indole-3-butyric acid
IGR	intergenic region
ILA	indole-3-lactate
Inr	initiator
iP	isopentenyl adenine
IPA	indole-3-pyruvic acid
iPDP	isopentenyl adenosine 5’-diphosphate
iPePP	isopentenyl pyrophosphate
iPMP	isopentenyl adenosine 5’-monophosphate
iPR	isopentenyl adenine riboside
iPRDP	isopentenyl adenine riboside 5’-diphosphate
iPRMP	isopentenyl adenine riboside 5’-monophosphate
iPRTP	isopentenyl adenine riboside 5’-triphosphate
Ipt	isopentenyl transferase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
iPTP	isopentenyl adenosine 5’-triphosphate
JA	jasmonic acid
kb	kilobase
kDA	kilodalton
l	litre
m-	milli-
MAPK	mitogen-activated protein kinases
MEKK	MAP kinase kinase kinase
MES	2-(N-morpholino) ethanesulfonic acid
MIG1	Multicopy Inhibitor of Galactose
min	minute
MINI3	MINISEED3

miRNA	micro RNA
MKK	MAP kinase kinase
MKS1	MPK4 substrate 1
mp	*monopteros*
MPK	MAP kinase
MR	middle region
mRNA	messenger RNA
n-	nano
NLS	nuclear localization signals
nph4	*nonphototropic hypocotyl 4*
nYFP	N-terminal half of YFP
OD	optical density
P value	probability value
PAA	phenylacetic acid
PAMPs	pathogen-associated molecular patterns
PCR	polymerase chain reaction
PM	Primer-Mix
Pmini	minimal promoter
PR1	pathogenesis-related genes 1
pro	promoter
PTA	protoplast transactivation
Q-rich	glutamine-rich
qRT PCR	Quantitative real-time PCR
rcf	relative centrifugal force
RD	repression domain
RNA	ribonucleic acid
rpm	rounds per minute
RT-PCR	reverse transcription PCR
s	second
SA	salicylic acid
SCF	Skp, Cullin, F-box protein
SD	Synthetic Defined medium
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SE	standard error
TAA1	tryptophan aminotransferase of *Arabidopsis*
TAFs	TBP-associated factors
TAM	tryptamine
TBP	TATA-binding protein
T-DNA	transferred-DNA
TDZ	N-phenyl-N'-(1,2,3-thidiazol-4-yl)urea thidiazuron
TEMED	tetramethylenediamine
TFII	TF indicates transcription factor; II indicates polymerase II
TFIID	TF, transcription factor; IID, RNA polymerase IID
Ti	tumor inducing

Ti Plasmid	tumor inducing plasmid
TIP	tumor-inducing principle
TIR1	transport inhibitor response 1
tml	tumor morphology large
tmr	tumor morphology rooty
tms	tumor morphology shooty
Trp	tryptophan
TSO	tryptophan side-chain oxidase
TSS	transcription start site
TTG2	TRANSPARENT TESTA GLABRA2
tZ	*trans*-zeatin
tZR	*trans*-zeation riboside
tzs	*trans*-zeatin synthesizing
UPM	Universal Primer A Mix
USER	Uracil-specific excision reagent
v	volt
VIP1	virE2 interacting protein 1
Vir	virulent protein
vir genes	virulence genes
WH	winged-helix
Y-1-H	yeast-one-hybrid
YFP	yellow fluorescent protein
YUC	YUCCA
ZMP	zeatinriboside 5’-monophosphate
β-ME	β-Mercaptoethanol
µ-	micro

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6.5 Declaration of independence

I hereby declare that my thesis entitled:

Regulation of Agrobacterial Oncogenes Expression in Host Plants

is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Wuerzburg

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