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Fang Fang

SIGNAL PERCEPTION AND TRANSMISSION THROUGH THE VIRA/VIRG TWO-COMPONENT SYSTEM IN AGROBACTERIUM TUMEFACIENS

By

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Doctor of Philosophy

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An Abstract of
A dissertation submitted to the Faculty of the Graduate
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Department of Chemistry
2007

Prokaryotes, fungi and plants sense and adjust to environmental change by two-component regulatory systems composed of HK (histidine kinase) and RR (response regulator) domains. Precise adaptation to complex environments often necessitates the integration of information from multiple cues. In *Agrobacterium tumefaciens*, the broad host range relies on the generality of signals and the integration of multiple input signals are regulated by the VirA/VirG two-component system. In this study, the VirA/VirG two-component system was used to explore the mechanism of signal perception and transmission and to reveal general strategies used for environmental sensing in prokaryotes.

VirA has been dissected into four domains, the periplasmic, linker, kinase and receiver domains. One striking feature is that VirA, as a hybrid kinase, carries an additional receiver at its C-terminus. The study of the role of the receiver domain in VirA/VirG activation indicated that the receiver domain encompasses both activating and inhibitory elements. With signal induction, the repression is relieved through dissociation of the linker/receiver interaction which makes the receiver to interact with the DNA binding domain of VirG for facilitating the phospho-transfer to the receiver domain of VirG. This may be a general mechanism for hybrid histidine kinases to integrate signals.

Another remarkable feature is that VirA, as a single receptor, detects multiple phenolic compounds. The short region aa288-293 in the VirA linker domain was found to regulate phenols specificity. Most importantly, single mutations such as Y293T in this region greatly reduce the range of known *vir* inducers. This short region therefore appears to be critical for the multi-host strategy developed by *A. tumefaciens* and has important practical consequences for the success of *Agrobacterium*-plant interaction.

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ABBREVIATIONS

aa	amino acid
Ab	antibody
acc	agrocinopin catabolism regulon
Ap	ampicillin
AS	acetosyringone
ASBr	α -bromo acetosyringone
bp	base pair
dd	double distilled
DTT	dithiothreitol
FDNB	2-4-dinitrofluorobenzene
x g	rcf or centripetal force of rotation
GBP	glucose binding protein
hr	hour
HSK	histidine sensor kinase
HDI	3-hydroxy-4, 6-dimethoxy-3H-isobenzofuran-1-one
I. M.	induction medium
IPTG	isopropyl β -D-thiogalactopyranoside
kan^r	kanamycin resistance gene
Km	kanamycin
kb	kilobases
kDa	kilo daltons

lac	lactose operon
LB	Luria-Bertani broth
LZ	leucine zipper
µg	micro gram
µl	micro liter
µM	micro molar
mg	milli gram
mL	milli liter
mM	millimolar
MES	2-(4-Morpholino)ethanesulfonic acid
min	minute
occ	octopin catabolism regulon
OD	optical density
PAGE	polyarylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
r	resistance
RBP	ribose binding protein
RR	response regulator
rt	room temperature
rpm	rotations per minute
SDS	sodium dodecylsulfate
Spec	spectinomycin

TBS	Tris buffered saline
T-DNA	transferred DNA
Tc	tetracyclin
Ti	tumor inducing
Tris	tris-(hydroxy methyl) aminomethane
vir	virulence regulon

CHAPTER 1

The *A. tumefaciens* VirA/VirG Two-Component System and Signal Perception

In the face of limited resources, bacteria are able to detect minute fluctuations in chemical resources and physical conditions for survival (Foussard *et al*. 2001). Among a variety of detection systems, two-component systems (TCSs) being predominant are used by bacteria to sense the environmental stresses and transduce the information inside cells (Bijlsma *et al*. 2003). These TCSs are abundant in most eubacteria in which they constitute about 1% of the encoded proteins (Table 1). In *E. coli*, there are 62 two-component proteins involved in regulating diverse processes that vary from chemotaxis to osmoregulation, metabolism and transport. Two-component systems also are present in both Gram positive and Gram negative pathogenic bacteria, some of which control expression of toxin production and pathogenesis (Bijlsma *et al*. 2003).

Two-component Paradigm

To facilitate information transfer within and between proteins, many signaling systems contain typical "transmitter" and "receiver" modules. TCSs contain a kinase sensor (transmitter) and a cognate response regulator (receiver). The kinase sensor monitors a particular environmental parameter and the response regulator mediates an adaptive response, e.g., through a change in gene expression (Eguchi *et al*. 2005).

Sensors typically contain a C-terminal transmitter coupled with an N-terminal

Table 1.1 Prevalence of two-component proteins in representative genomes [a] (Bijlsma *et al*. 2003)**.**

Organism	HKs		RRs	HPt proteins[b]
	Orthodox	Hybrid		
Escherichia coli	24	5	32	1
Synechocystis sp	25	16	38	1
Streptococcus pneumoniae	13	0	14	ND
Methanobacterium thermoautotrophicum	14	1	9	ND
Saccharomyces cerevisiae	0	1	2	1
Dictyostelium discoideum[c]	0	11	1	1
Arabidopsis thaliana	2	9[d]	16	5

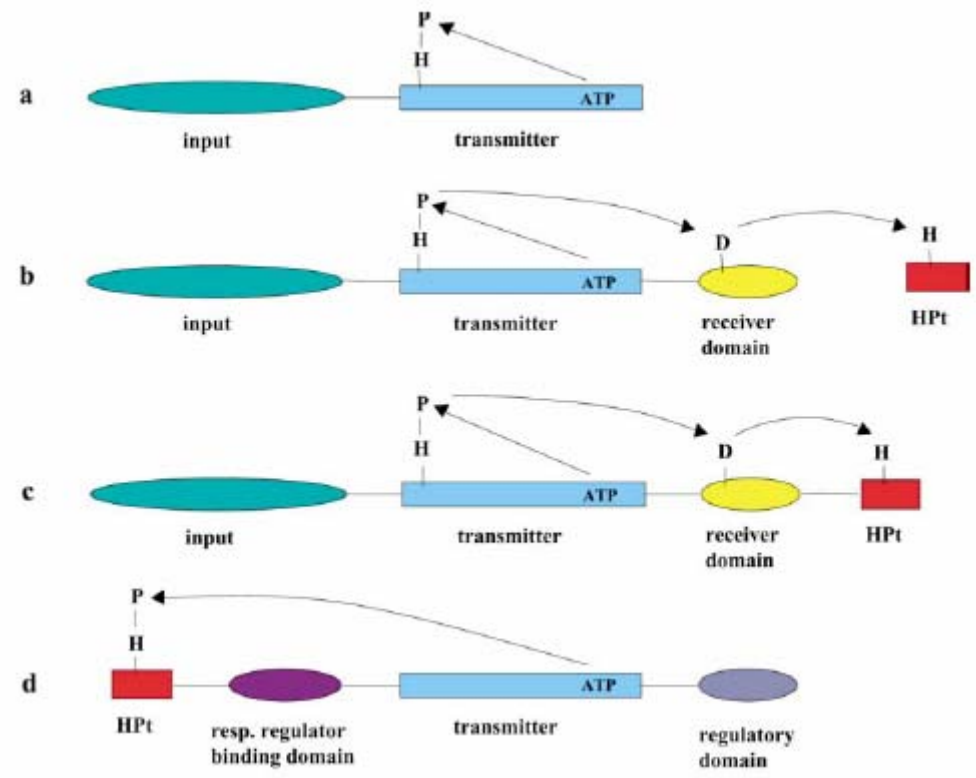
[a]Abbreviations: HK, histidine protein kinase; HPt, His-containing phosphotransfer; ND, not determined; RR, response regulator protein.
[b]Independent of domains, not part of HKs.
[c]Genome sequence is not complete.
[d]Six out of nine in this category are documented as being hybrid HKs; the other three are unconfirmed.

signal input domain. Response regulators contain an N-terminal receiver module coupled with a C-terminal output domain. In the presence of a stimulus, the communication between kinase sensors and response regulators involves phosphate transfer. The kinase sensor is autophosphorylated at histidine and the phosphoryl group is transferred to an aspartyl group in the response regulator to trigger the downstream response. This simplified picture may have layers of complexity, depending on whether the sensor exhibits phosphatase activity towards its own phosphorylated form or towards that of the phosphorylated response regulator. More complex 'phosphorelay' systems can also involve additional phospho-transfer proteins as shown in Fig 1.1, opening opportunities for even greater regulatory control (Appleby *et al*. 1996; Cavivhiolo *et al*. 1995; Diep *et al*. 2001; Kreikemeyer *et al*. 2001).

Histidine Sensor Kinase

The characteristic histidine sensors are around 350 amino acids in length and have fingerprints termed homology boxes, the H-, N-, D-, F-, and G-boxes (Stock *et al*. 2000; Parkinson and Kofoid, 1992). The H-box contains the site of histidine phosphorylation while N, G1, F and G2 boxes constitute the catalytic ATP-binding domain. HSKs generally exist as dimers and assembly as four-helix bundles that are believed to phosphorylate the conserved His residues of their partners (Wise *et al*. 2005). The N-terminal sensing domains are believed to determine the rate of autophosphorylation of the histidine kinases (Foussard *et al*. 2001). The diversity of sensing domains suggests specific ligand/stimulus interactions, however, the regulation of early signaling steps and even definition of the exact molecular signals have been difficult to characterize. In fact,

Fig 1.1 Representative histidine kinases (Foussard *et al*. 2001)**.** (a) kinase contains a variable length input domain able to sense a variety of signals. (b) kinase contains input domain, an additional receiver domain and a separate phospho-transfer unit (HPt). (c) kinase contains input domain, an additional receiver domain and a covalently attached phosphotransfer unit (HPt). (d) kinase contains an input domain coupled to membrane receptors. Green, input domain; light blue, ATP binding domain; yellow, receiver domain; red, HPt domain; purple, response regulator binding domain; blue, regulatory domain.



the possible role played by multiple signals on any particular two-component system has been rarely examined. One exception is the NO responsive sensor kinase ResE of *Bacillus subtilus* (Baruah *et al*. 2004) where genetic evidence suggests multiple signal inputs, one recognized in the cytoplasm and one in the periplasm. Similarly, recent *in silico* analysis of BvgS (the sensor kinase regulating pathogenesis in *Bordetella pertussis*) indicates two distinct periplasmic protein-binding domains and a cytoplasmic PAS domain (Camille *et al*. 2006), all theoretically capable of responding to separate signals. Therefore, a HSK with well-characterized signals would be important for understanding the kinase activation mechanism.

Response Regulator

Compared to the HSKs, the RRs are better characterized in terms of structure and function. The response regulators share a common phosphorylatable receiver domain and some carry additional functional modules whose activity may be regulated by the receiver as shown in Fig 1.2 (Foussard *et al*. 2001). The receiver domains in RRs display a doubly wound α/β fold with a central five-stranded β sheet surrounded by five α helices. Phosphorylation of the conserved Asp residue leads to a conformational change involving a large surface of the RR that facilitates the inter- or intra-molecular interaction described in Fig 1.3 (Robinson *et al*. 2003). It has been suggested that the phosphorylation-induced conformational change can promote either the dimerization of the effector domain or release the effector domain from interacting with its own receiver domain to activate gene transcription. Structural homology of RRs provides valuable comparisons with other members of the family.

Fig 1.2 Modular organization of the response regulators (Foussard *et al*. 2001)**.** These proteins share common phosphorylatable receiver domains which regulate additional functional modules such as DNA-binding, ATPase or other regulatory domains. Yellow, receiver domain; purple, DNA-binding domain; light blue, enzymatic domain; pink, ATPase domain; grey, regulatory domain.

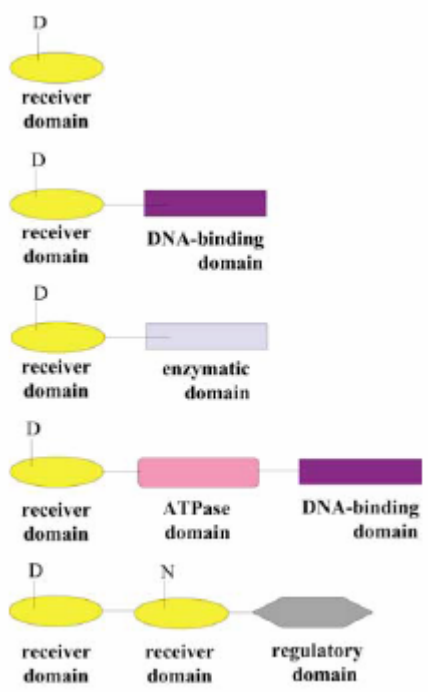
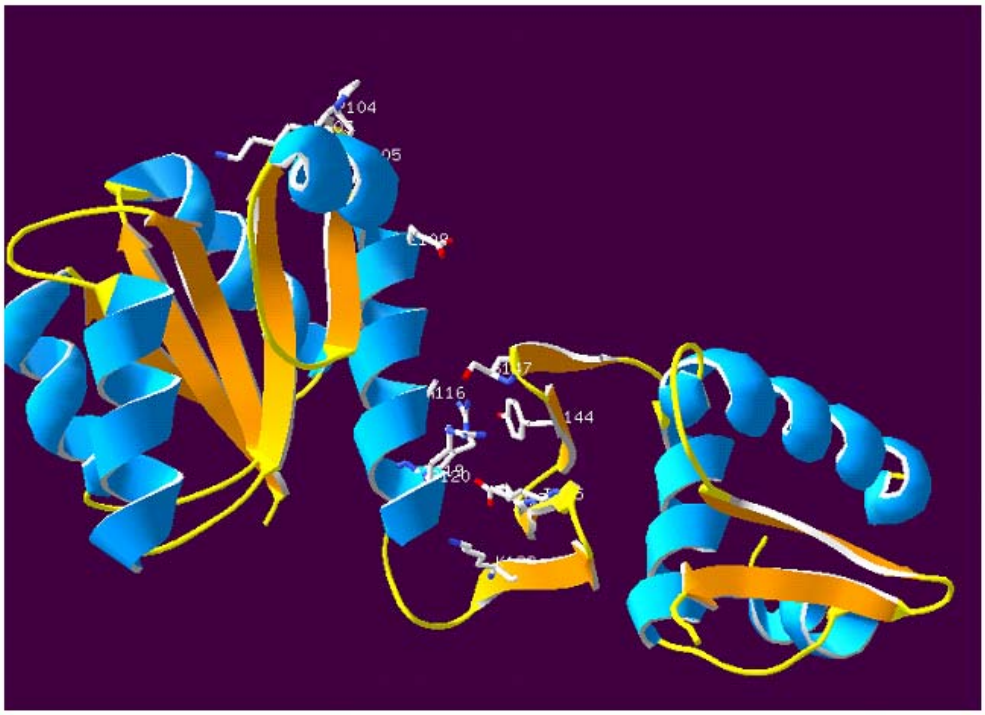


Fig 1.3 Structure of the response regulator DrrB of the DrrA/DrrB two-component system (Robison *et al*. 2003)**.** DrrB contains two domains, a receiver domain with five α-helices and five β-strands and DNA-binding domain with winged-helix. These domains are connected by a flexible loop which includes four β-strands packed against the α5 helix of the receiver. Residues involved in the important interactions between α5 helix in the receiver and the flexible loop are shown in stick representation: Blue, α-helices; yellow, β-strands; grey, amino acid residues.



The VirA/VirG two component system of *Agrobacterium tumefaciens*

Agrobacterium tumefaciens and Crown Gall Disease

Agrobacterium tumefaciens can cause plant tumors by transferring fragments of oncogenetic DNA to host plant cells. (Heath, Charles *et al*. 1995; Zhu, Oger *et al*. 2000; Tzfira and Citovsky 2002). This lateral gene transfer represents a rare example of inter-kingdom gene transfer and has been successfully extended and exploited to generate transgenic plants (Binns *et al*. 2002). The molecular mechanisms of the gene transfer have been studied in detail and our current understanding is illustrated in Fig 1.4. The genes mediating pathogenesis are located on a 200 kb mega plasmid known as the tumor-inducing (Ti) plasmid, and they are located in five regions: (i) the T region, which codes the sequences that are transferred to the plant host; (ii) the *vir* region, which directs the processing and transfer of the T-DNA; (iii) the *rep* region, which is required for replication of the Ti plasmid; (iv) the *tra* and *trb* loci, which direct the conjugal transfer of the Ti plasmid; and (v) genes that direct uptake and catabolism of opines (Zhu *et al*. 2000). The pathogenesis is initiated when *Agrobaterium tumefaciens* strains are exposed at the wound sites of plants where chemical signals including sugar, acidic pH and phenolic compounds are secreted. The VirA/VirG two-component system directs the phosphorylation of VirG which activates expression of the *vir* regulon. These genes all play predicted roles in facilitating T strands integration into the host genome. For examples, VirD cleaves double strand T-DNA to single T-strands; VirB includes many protein components which assemble as a pore to deliver single T-strands and VirE2 mediates transport of the T-DNA from the cytoplasm to the plant nucleus. Oncogenic proteins encoded in the T-DNA produce plant hormones, auxin and cytokinin, leading to

the uncontrolled growth of plants cells.

VirA/VirG Two-Component System

The VirA/VirG two-component system is therefore the key for initiating pathogenesis. As shown in Fig 1.5, VirA is thought to function as a membrane bound homodimer (Melchers *et al*. 1989; Pan *et al*. 1993; Winans *et al*. 1989), and the importance of dimerization for VirA activity is indicated by experiments showing that ATP bound to one subunit is utilized to phosphorylate the other subunit within the dimer (Brencic *et al*. 2004; Toyoda-Yamamoto *et al*. 2000; Wise *et al*. 2005). In fact, signals resulting from both phenol and sugar perception can be transferred from one monomer to the other (Toyoda-Yamamoto *et al*. 2000; Wise *et al*. 2005). Each VirA monomer within a dimer consists of distinct functional domains (Winans *et al*. 1989; Chang *et al*. 1992; Melchers *et al*. 1989). Two transmembrane regions define an N-terminal periplasmic (P) domain. The cytoplasmic portion of VirA consists of three additional regions: the linker (L), the kinase (K), and the receiver (R). The periplasmic domain is involved in sugar signaling through interaction with ChvE (Shimoda *et al*. 1993); the kinase domain contains the conserved histidine that is the site of autophosphorylation (Jin *et al*. 1990) and an ATP-binding site (Parkinson *et al*. 1992); and the receiver domain has some homology with the N-terminus of the VirG response regulator. One of the most intriguing but amazing features of the VirA molecule is that several of its domains can function when expressed separately (Gao *et al*. 2005) and these have been used to develop the model shown in Fig 1.5.

Recent evidence suggests that the periplasmic domain detects sugar and pH through another protein ChvE; however, considerable debate exists as to whether phenol

Fig 1.4 Two-way exchange of chemical signals between *A. tumefaciens* and host plants (Zhu *et al*. 2000)**.** Wound-released chemical stimuli are perceived by the VirA proteins, which lead to transcription of *vir* promoters through VirG activation. T-DNA is processed by the VirD2 protein, and single-stranded linear T strands are formed by strand displacement. T strands and VirE2 are translocated from the bacteria via a pore encoded by the *virB* operon and form a T complex within the plant cytoplasm. T complexes are transported into the nucleoplasm via the host protein karyopherin alpha, and the T-DNA is integrated into genomic DNA. Transferred genes encode phytohormone synthases that lead to plant cell proliferation and opine synthases, molecules that provide nutrients to the colonizing bacteria. Opines are released from the plant cell, enter the bacteria via dedicated opine permeases, and are catabolized via opine-specific catabolic proteins. Opine permeases and catabolic enzymes are encoded on the Ti plasmid.

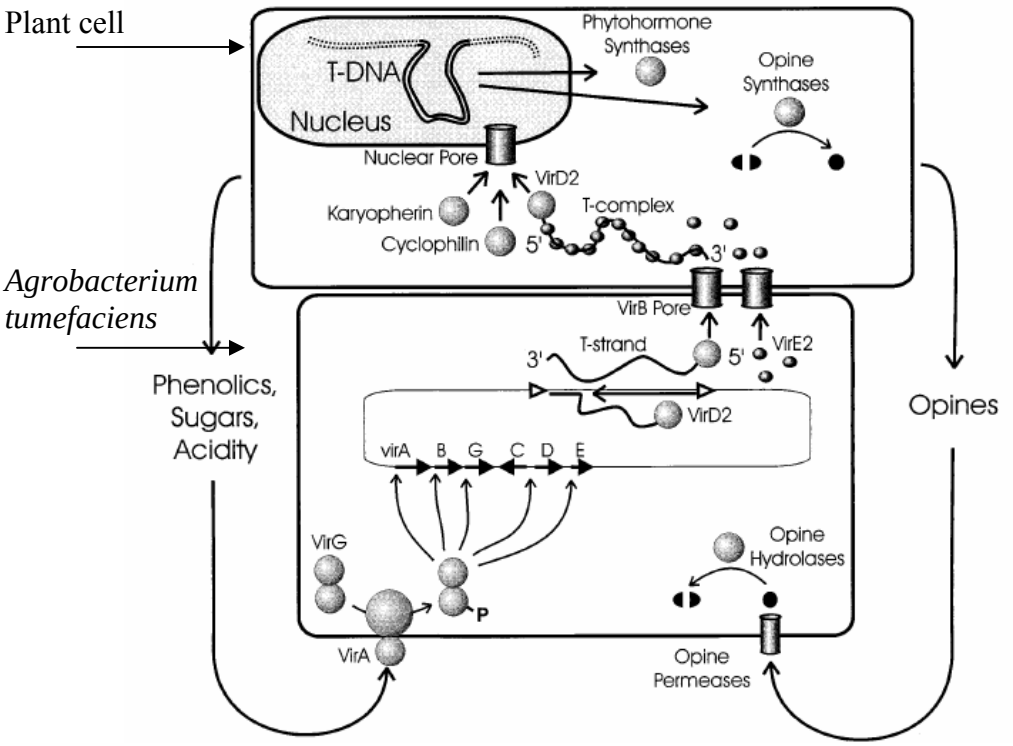
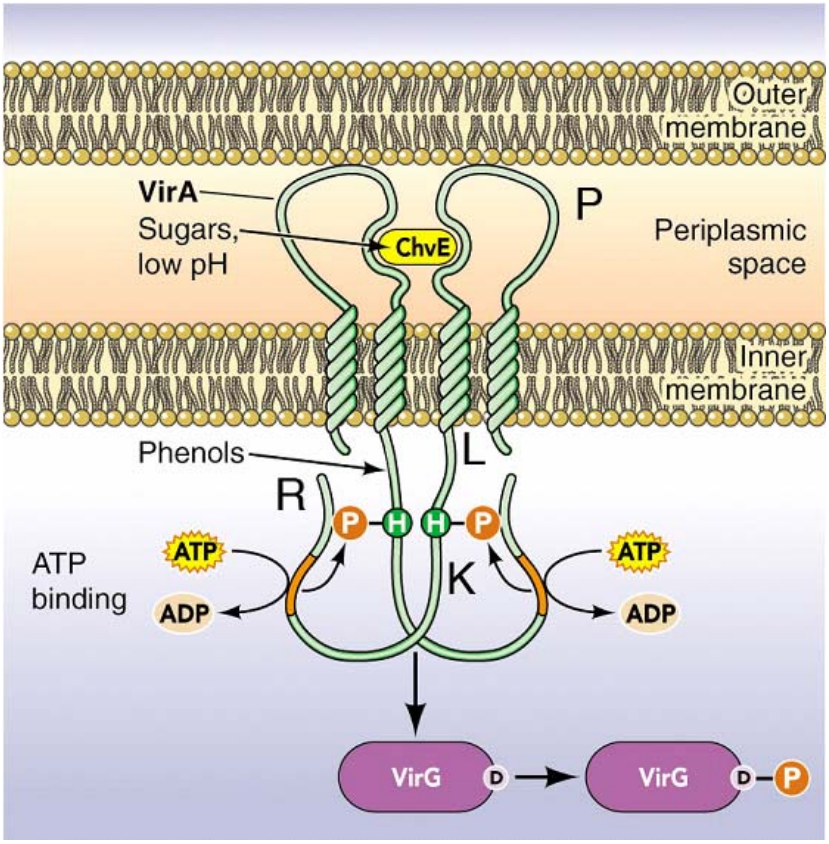

Plant cell
Agrobacterium tumefaciens
Phenolics, Sugars, Acidity
Opines
T-DNA
Nucleus
Nuclear Pore
Phytohormone Synthases
Opine Synthases
VirD2
Karyopherin
Cyclophilin
T-complex
5'
3'
VirB Pore
T-strand
VirE2
virA
B
G
C
D
E
VirG
P
VirA
Opine Hydrolases
Opine Permeases

Fig 1.5 Schematic representation of VirA/VirG function. VirA forms membrane bound homodimers, and ATP bound to one subunit is utilized to phosphorylate the other subunit within the dimer. Each VirA monomer within a dimer consists of distinct functional domains**.** Two transmembrane regions define an N-terminal periplasmic (P) domain. The cytoplasmic portion of VirA consists of three additional regions: the linker (L), the kinase (K), and the receiver (R). The periplasmic domain is involved in sugar signaling through interaction with ChvE; the kinase domain contains the conserved histidine that is the site of autophosphorylation and an ATP-binding site; and the receiver domain has some homology with the N-terminus of the VirG response regulator.



perception follows the model. Two chromosomal proteins, P10 and P21, have been proposed to interact directly with phenolic compounds; an interaction that would drive a conformational change of VirA for activation (Lee *et al*. 1992). Other data argues that VirA itself acts as the direct sensor for phenolic compounds ((Lee *et al*. 1996; Lohrke *et al*. 2001). In Chapter 2, we extend this study and find further support for VirA serving as the direct sensor for phenols.

Based on the functional analysis of different domains of VirA, the conserved kinase domain (K) functions for phospho-transfer to VirG, the periplasmic domain (P) is responsible for sensing low pH/monosaccharides, the linker domain (L) is involved in phenol perception (Chang *et al* 1992; Gao *et al* 2005) and the additional C-terminal receiver domain (R) appears critical for signal regulation (Chang *et al* 1996; Jin *et al* 1990; Mukhopadhyay *et al* 2004).

However, both the current lack of structural information and low homology of the linker sequences with known structures, complicate mechanistic insight into phenol activation. In most histidine kinases, dimeric association is a key for their functions. VirA, as with other histidine kinases, would form a four-helix bundle within the cell membrane (Pan *et al* 1993). More recently, two helical coiled coils, Helix-C and Helix-D, has been identified by PHD-sec algorithms in the VirA linker domain. Helix-C is at the N-terminus of the linker domain, connecting the periplasmic domain via TM2, and Helix-D is at the C-terminus of the linker domain, extending the helix into the kinase domain through His474. Fusions between these domains and the leucine zipper (LZ) of GCN4 have been shown to activate/suppress the phenol response (Wang *et al* 2002). The resulting "ratchet" model that this data supports posits that signal perception causes the ratcheting

of interfaces between Helix-C dimers and this signal-induced switching of interfaces corresponds to a rotational motion between dimer subunits that propagate through the kinase domain to mediate phosphorylation. In Chapter 3, a novel function of the linker domain has been found in which the linker interacts with the receiver domain to relieve repression.

Phenolic compounds are key inducers to activate VirA/VirG while acidic pH and monosaccharide only act synergistically with the phenolic compounds (McCullen *et al*. 2006). *Agrobacterium tumefaciens* infects at wound sites of a wide range of hosts, including over 300 genera of dicotyledonous plants, at least four families of monocots, and over 40 species of gymnosperms. In contrast, host recognition by *Agrobacterium*'s close relative, *Rhizobium spp.*, is initiated by a unique flavanoid released by its specific legume host to establish a pair-wise rhizobia/legume symbiotic relationship (Magne *et al*. 1995). In the multi-host pathway, the host signals represent the general features of most plant wound sites, and the structural features of phenolic compounds have been analyzed by testing the *vir*-inducing ability of around fifty compounds (Melchers *et al*. 1989; Duban *et al*. 1993). In Chapter 4, we describe a novel feature of the region at the N-terminus of the linker domain which changes the phenol specificity related with acetyl groups in the para position. This region, aa288-aa293 in the VirA linker domain, is assigned as responsible for phenol specificity and single mutations can be sufficient to switch specificity. This short region therefore appears to be critical for the multi-host strategy developed by *A. tumefaciens* and has important practical consequences for the success of *Agrobacterium*-plant interaction.

VirA carries an additional C-terminal domain with sequence similarity to the

receiver domain of the response regulators. The presence of this receiver domain defines VirA as a hybrid kinase. The function of VirA's receiver appears to differ from those carried on some other hybrid kinases in a number of ways. First, VirA does not appear to become phosphorylated on D766, the residue analogous to Asp52 on the VirG receiver domain (Mukhopadyhay *et al*. 2004) nor it is required for activity (Pazour *et al*. 1991). Second, VirA does not include an additional HPT domain. While, we cannot exclude the possibility of a separate HPT protein assisting in transfer of the phosphate from VirA to VirG, there is no genetic evidence for this domain. In fact, purified VirA rapidly phosphorylates purified VirG, suggesting that intervening phospho-relay proteins are not required for VirG activation (Jin *et al*. 1990). Finally, previous analyses of VirA demonstrates that removal of the receiver allows *vir* gene expression in the absence of a phenolic inducer, provided sugar and acidic pH are present (Chang *et al*. 1992; Chang *et al*. 1996; Brencic *et al*. 2004; Gao *et al*. 2005). These experiments led to the description of the VirA receiver domain as an inhibitory element. In contrast to the studies described above, the experiments presented in Chapter 5 indicate that VirA's receiver domain behaves as an enhancing element. The VirA receiver may function as a recruitment and/or alignment factor to effectively increase the availability of VirG for phosphate transfer from VirA's kinase region to the VirG receiver domain, suggesting a role not previously considered for the receiver domains of hybrid kinases.

The VirA/VirG two-component regulatory system then provides the first and now the best described model for signal perception and integration among two-component systems. The understanding of the signaling mechanisms reveals the general principles of how “information” is processed, integrated and translated during pathogenesis and has

the potential to reveal important information about biological signaling generally.

CHAPTER 2

Phenolic Activation of *A. tumefaciens* Virulence Genes: Exploiting Heterologous Hosts to Define Crucial Components

Introduction

At the time I began my Ph. D studies, there existed two competing hypotheses as to how phenolic signals were perceived by VirA. First, the perception event involved separate phenolic receptors that bind the phenol and drove the conformational change for VirA activation. The supporting evidence included the diverse structures being recognized were not consistent with a single receptor and AS hypersensitive mutants were found not to be located in Ti plasmids (Campbell *et al*. 2000). Moreover, two chromosomally proteins, P10 and P21 were found to physically bind to phenols (Lee *et al*. 1992). However, the roles of P10 and P21 have not been defined and it remains possible that the hypersensitive phenotype is the result of increased uptake or altered metabolism of the phenolic compounds.

Secondly, VirA itself was proposed to directly sense phenolic compounds. Support for this hypothesis came from the observation that the *virA* locus, not the *Agrobacterium* strain, determines which phenolic compounds act as inducers and that VirA/VirG is functional in the heterologous host *E. coli*, which presumably does not contain these additional proteins (Lee *et al*. 1996; Lohrke *et al*.2001). However, in *E. coli*, the activation of VirA/VirG is much lower than that in *A. tumefaciens* and no physical evidence exists for a direct interaction between phenolic compounds and VirA.

I decided to test these hypotheses biochemically. When VirA/VirG and the *A. tumefaciens rpoA* gene, the α-subunit of the RNA polymerase complex, are moved into *E. coli*, the system should be equally sensitive to the range of inducers and inhibitors that have been discovered for this system. Two natural products, DIMBOA, identified from maize root homogenate, and HDMBOA, identified from maize root exudates, have also been identified as specific inhibitors for *vir* gene induction (Zhang *et al*. 2000; Maresh *et al*. 2006). Moreover, a stable analog of HDMBOA, known as HDI (Maresh *et al*. 2006) and a mechanism based inactivator ASBr (Lee *et al*. 1992) have been developed. ASBr and HDI are powerful inhibitors for VirA/VirG two-component system in *A. tumefaciens*.

Combined with the previous reports that VirA/VirG is active in the heterologous system *E. coli* and inhibitors that have been constructed to compete with the inducer AS, I proposed to utilize these inhibitors to test the function of VirA/VirG in *E. coli*. The findings presented here confirm that the inducer AS and inhibitors have the same basic acting in *vir* induction both in *A. tumefaciens* and *E. coli*. I have therefore concluded that VirA functions as the direct sensor and further discussed the reasons for the lower activity in *E. coli*.

Material and methods

Bacterial Strains, Plasmids and Reagents. The bacterial strains and plasmids used in this study are listed in Table 2.1. *E. coli* strains XL1-Blue, XL10-Gold and DH5α were used for routine cloning. Acetosyringone (AS) used for *vir* induction assays was purchased from Sigma-Aldrich Corp. HDI and ASBr were synthesized in our lab. The

enzymes for DNA manipulation were purchased from either New England Biolab or Promega unless specified.

Table 2.1 Bacterial strains and plasmids used in Chapter 2

Strains /plasmids	Relevant characteristics	Reference
E. coli		
XL1-Blue	*recA1 endA1 gyrA96 thi¯1 hsdR17 supE44 relA1 lac[F' proAB lacI^{q}Z M15 Tn 10* (Tc^{r})*]*	Stratagene
XL10-Gold	Tet^{r} *(mcrA)183 (mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F' *proAB lacI^{q}Z M15 Tn10* (Tet^{r}) Amy Cam^{r}]^{a}	Stratagene
DH5α	*recA1 endA1 hsdR17 supE44 gyrA96 relA1Δ(lacZYA-argF) U169 (ϕ 80dlacZΔM15)*	Invitrogen
A .tumefaciens		
A136	Strain C58 cured of pTi plasmid	(Watson *et al*. 1975)
Plasmids		

Strains /plasmids	Relevant characteristics	Reference
pYW15b	Broad-host-range expression vector, IncW, Ap^r	(Wang *et al*. 2000)
pYW47	P_{N25}-6xHis-*virG* in pYW15b, IncW, Ap^r	(Wang *et al*. 2000)
pRG109	P_{N25}-6xHis-*virG* in pJZ4, $Spec^r$	(Gao *et al*. 2005)
pYW21	P_{N25}-6xHis-*virA(aa285-829)* in pYW15, Ap^r	(Wang *et al*.1999)
pRG119	P_{N25}-6xHis-*virA(aa426-711)* in pYW15, Ap^r	(Gao *et al*. 2005)
pFF4	P_{N25}-6xHis-*virA* in pYW15b, Ap^r	This chapter
pFQ13	pYW15b without pBR322 origin, Ap^r	This chapter
pFQ19	P_{N25}-6xHis-*virG* in pFQ13, Ap^r	This chapter
pFQ25	P_{N25}-*virA* and P_{N25}-6xHis-*virG* in pFQ13, IncW, Ap^r	This chapter
pPS1.3	P_{lac}-*rpoA* from *A. tumefaciens* A136 in pTZ19R, Ap^r	(Lohrke *et al*. 2001)
pET1	P_{lac}-*rpoA* from *A. tumefaciens* A136 in $pET24a^+$, Kan^r	This chapter

Strains /plasmids	Relevant characteristics	Reference
pBRR122	Broad-host-range expression vector with medium copy numbers origin, Cm^r and Kan^r.	Boca Scientific
pFQ108	*virB::lacZ* in pBRR122, Kan^r	This chapter
pFQ111	P_{N25}-6xHis-*virG, virB::lacZ* in pBRR122, Kan^r	This chapter
pAM13	P_{N25}-6xHis-*virG(N54D)* in pYW15b, Ap^r	(Mukhopadhyay, 2002, thesis)
pFQ100	P_{N25}-6xHis-*virG(N54D), virB::lacZ* in pBRR122, Kan^r	This chapter
pFQ114	P_{N25}-6xHis-*virA*(426-711), P_{N25}-6xHis-*virG, virB::lacZ* in pBRR122, Kan^r	This chapter
pFQ115	P_{N25}-6xHis-*virA*(285-829), P_{N25}-6xHis-*virG, virB::lacZ* in pBRR122, Kan^r	This chapter

Cloning, Transformation and Growth Conditions. *E. coli* strains were grown in Luria-Bertani (LB) medium at 37 °C overnight. *A. tumefaciens* strains were grown in LB

medium, or induction medium (I. M.) (Cangelosi *et al*. 1991) containing 1% glycerol at 28°C. Plasmid DNAs were purified using GeneElute Plasmid Miniprep kit from Sigma. DNA subcloning was carried out following the standard procedures. Plasmids or PCR products were digested with restriction enzymes and then separated by 0.8~1.5% agarose TBE gels. The desired fragments were purified by either Qiaquick gel purification kit (Qiagen) or Perfectprep gel cleanup kit (Eppendorf). Ligation was usually carried out in a 15 μL volume at 14°C-16°C for 10-14 hours. Transformations into *E. coli* strains were performed by either heat-shock or electroporation methods following the protocols provided with the competent cells. Alternatively, *E. coli* competent cells were prepared and transformed as described (Ausbuel *et al*. 1995). To prepare electrocompetent cells of *Agrobacterium tumefaciens*, 50ml bacteria grown to OD_{600} 0.4-0.6 were resuspended in 50 mL autoclaved distilled water plus 10% glycerol and washed for 3~4 times. Transformations into *A. tumefaciens* were done by electroporation using the Gene Pulser II (Bio-Rad) with the following condition: 2.4 kV, 400 ohms (Pulse Controller Unit) and 25κF (capacitor). After the transformation, 950 μL of LB at room temperature was added to the transformed cells immediately and the cells were recovered at 28°C for 2 hours prior to the plating on LA agar plates with appropriate antibiotics.

Plasmid constructions. The plasmids used in this study are listed in Table 2.1. The coliphage T5 promoter (P_{N25}) was used to drive the expression of *virG* independent of environmental factors like pH and phosphate concentration (Wang *et al*. 2000). The Nco I fragment containing P_{N25}-6xHis-*virG* from pYW47 was blunt-ended with Klenow fragment and ligated with pJZ4 that was treated with Kpn I and Klenow fragment, resulting in pRG109 (Gao *et al*. 2005).

To make a shuttle vector with a single origin, pYW15b was digested by Drd I and self-ligated to generate pFQ13 (low copy number plasmid). P_{N25} driven *virG* from pYW47 was treated with EcoR I and Kpn I and inserted into pFQ13 to make pFQ19. The coding region of *virA* gene was placed behind P_{N25} and inserted into pRG109 using Kpn I cutting sites to create pFF4 and P_{N25}-*virA* was subsequently released to insert into pFQ19 to give pFQ25.

To insert soluble VirA truncations fragments in the shuttle vector with higher copy number origin, the BamH I fragment containing *virB::lacZ* from pRG109 was blunt-ended with Klenow fragment and ligated with pBRR122 shown in Fig 2.2 that was treated with Dra I and Alkaline Phosphatase, resulting in pFQ108. P_{N25}-6xHis-*virG* from pYW47 and P_{N25}-6xHis-*virG* (N54D) from pAM13 were digested with Nco I and inserted into pFQ108 at the same restriction cutting site to create pFQ111 and pFQ100. To make plasmids pFQ114 and pFQ115 shown in Fig 2.1, plasmids pYW21 and pRG119 were digested with Nco I and blunt-ended with Klenow fragment and ligated with pFQ111 that was treated with Kpn I and Klenow fragment.

Plasmid pPS1.3 containing *rpoA* gene from *A. tumfaciens* was digested with Sap I and BamH I and blunt-ended with Klenow fragment at the Sap I cutting site. The fragment was inserted into pET24a+ that was treated by Sph I and BamH I and blunt-ended with Klenow fragment at the Sph I cutting site to give plasmid pET1.

β-galactosidase assays for *vir* gene induction. Cells were grown in 20 mL of LB medium to OD_{600} 0.4-0.6 in the presence of the appropriate antibiotics. The cell mass was pelleted by centrifugation for 10 min at 7000 x *g* at 4°C and washed with PBS. The

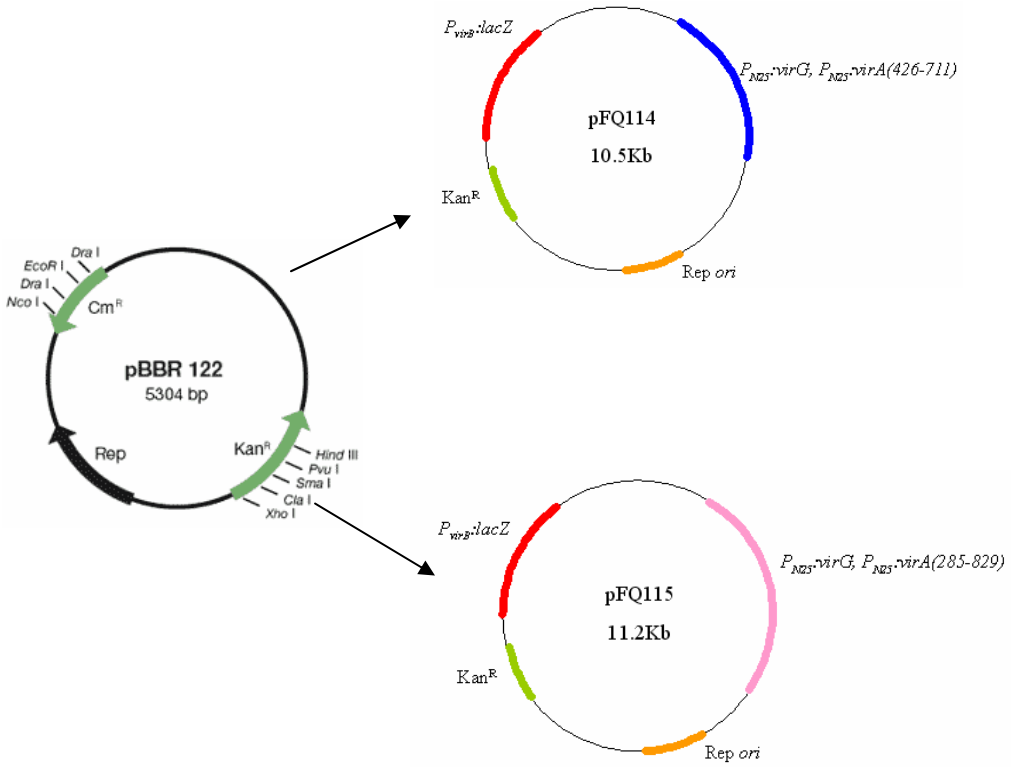
pellet was diluted to OD_{600} of ~ 0.1 into tubes containing a total of 1 mL of induction media (I.M) (Cangelosi *et al*. 1991) and cultured at 28^{o}C with shaking at 225 rpm for 15 hrs. β-galactosidase activity was determined as described (Miller 1972) with readings of optical densities at 600 nm and 415 nm using the EL800 microplate reader (BIO-TEK Instruments). For β-galactosidase assays with inhibitors, the induction media (I.M) were supplemented with desired concentrations of inhibitors and 100 μM AS. 1% glycerol was used as the carbon source and the cells were incubated in I.M. at 28°C for 8 hrs before assaying for β-galactosidase.

Results

VirA condition monitoring for *vir* induction both in *A. tumefaciens* and in *E. coli*

The broad-host-range expression system based on P_{N25} is able to productively express protein both in *E. coli* and *A. tumefaciens*. Accordingly, P_{N25} allows for VirG expression under nonacidic and nonstressful conditions and avoids autocatalytic overexpression in *A. tumefaciens*. To ensure VirA/VirG function in the heterologous *E. coli* host and to simplify the the complex transcriptional regulation of VirG, P_{N25}-6xHis-VirA, P_{N25}-6xHis-VirG and P_{virB}-lacZ were inserted in shuttle vector pYW15b to give the plasmid pFF4.Together with the plasmid pPS1.3 which has *rpoA* gene, the α-subunit of the RNA polymerase complex, from *A. tumefaciens*, pFF4 was transformed into *E. coli* strain DH5α. However, the VirA/VirG construct proved not to be active in *E. coli*. We reasoned that the vector pYW15b, which has two origins, IncW and pBR322, may cause spontaneous errors for DNA replication in *E. coli*, or the six histidine residues at the N-

Figure 2.1 Physical maps of the Broad-host-range expression vector pBBR122 and the derivatives containing P_{N25}-6xHis-*virG*, *virB::lacZ*, and P_{N25} driven VirA alleles.



terminus of VirA may result in improper localization in *E. coli*. To rule out these potential factors, plasmid pFQ25, a low copy number plasmid with only the IncW replication origin, containing P_{N25}-VirA, P_{N25}-6xHis-VirG and P_{virB}-lacZ was constructed and was transformed into *A. tumefaciens* strain A136. Together with the plasmid pET1 containing *rpoA* gene from *A. tumefaciens*, pFQ25 was also transformed into *E. coli* strain DH5α. Fig 2.2 shows that with AS, VirA and VirG are functional both in *A. tumefaciens* and in *E. coli*, although the activity is significantly lower in *E. coli*. Although the maximal activity in *A. tumefaciens* is about seven fold of the maximal activity in *E. coli*, compared to *vir* induction with and without AS, the level of the *vir* induction with AS in *E. coli* and *A. tumefaciens* is similar, suggesting AS should be detected by the same proteins to activate VirA/VirG two-component system both in *A. tumefaciens* and in *E. coli*.

VirA localization is not the factor for lower activity of VirA/VirG in *E. coli*

VirA is a transmembrane protein, likely leading to protein misfolding or improper localization in the heterologous *E. coli* system. To test this possibility, P_{N25} driven VirA truncations, VirA(285-829) and VirA(426-711), as mapped in Fig 2.3A were transformed into *E. coli* with the *rpoA* gene from *Agrobacterium* and the *virB::lacZ* reporter system. As shown in Fig 2.3B, 200μM AS still induce the expression of VirA(285-829) or VirA(426-711) in *E. coli*. The level of the activity is comparable, but the background is high, giving a lower level of induction. Nevertheless, these data indicates that VirA localization in *A. tumefaciens* does not play a significant role for lower activity of VirA/VirG in *E. coli*.

Figure 2.2 P_{virB}-*lacZ* expressions by P_{N25}-VirA/ P_{N25}-6xHis-VirG in both *A. tumefaciens* and *E. coli* strains. *A. tumefaciens* A136 containing pFQ25 and *E. coli* DH5α containig pFQ25 and pPS1.3 were cultured for 12 hrs at 28°C in the medium supplemented with 1% glycerol and 200μM AS.

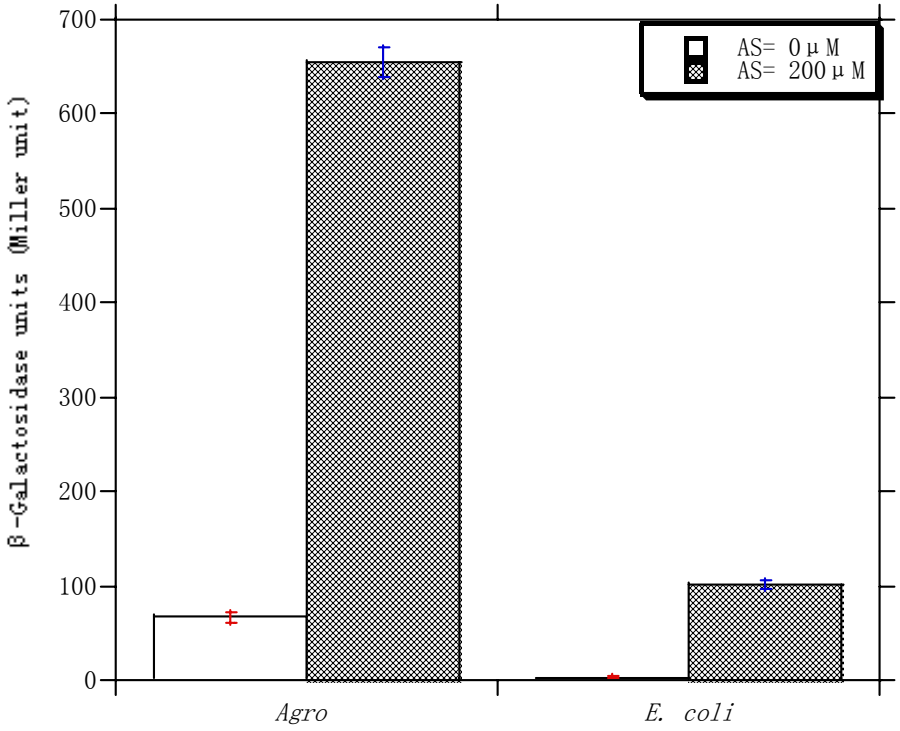
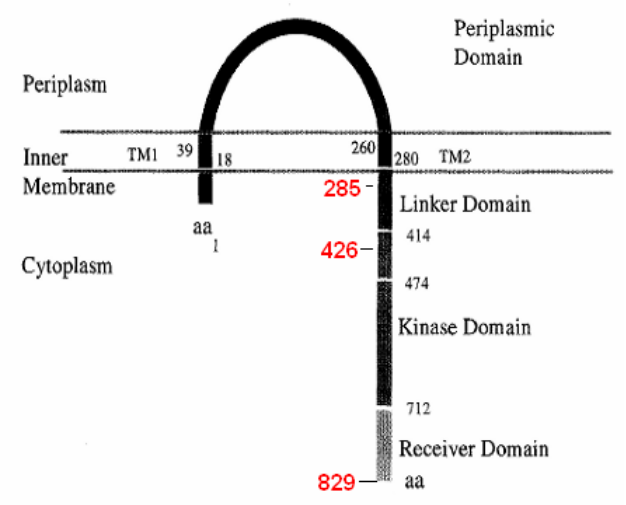
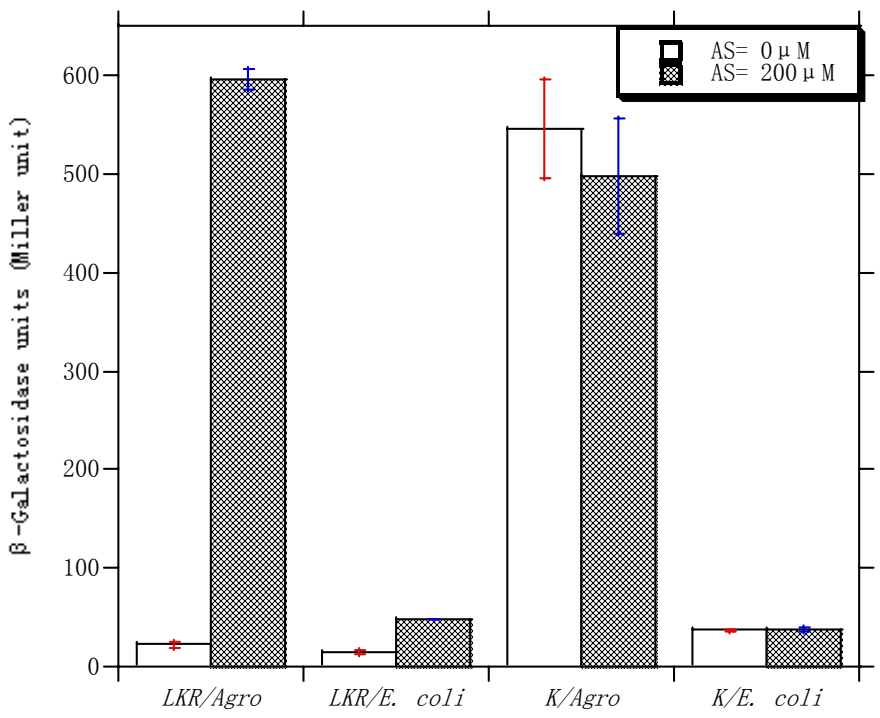


Figure 2.3 P_{virB}-*lacZ* expressions by P_{N25}-VirA truncations/ P_{N25}-VirG in both *A. tumefaciens* and *E. coli.* (A) Schematic representation of VirA domains (Lee *et al.* 1996). (B) *A. tumefaciens* A136 containing pFQ114 or pFQ115 and *E. coli* DH5α containig pFQ114 / pPS1.3 or pFQ115 / pPS1.3 are evaluated with and without 200 μM AS.

(A)



(B)



Probing specificity: Inhibitors ASBr and HDI

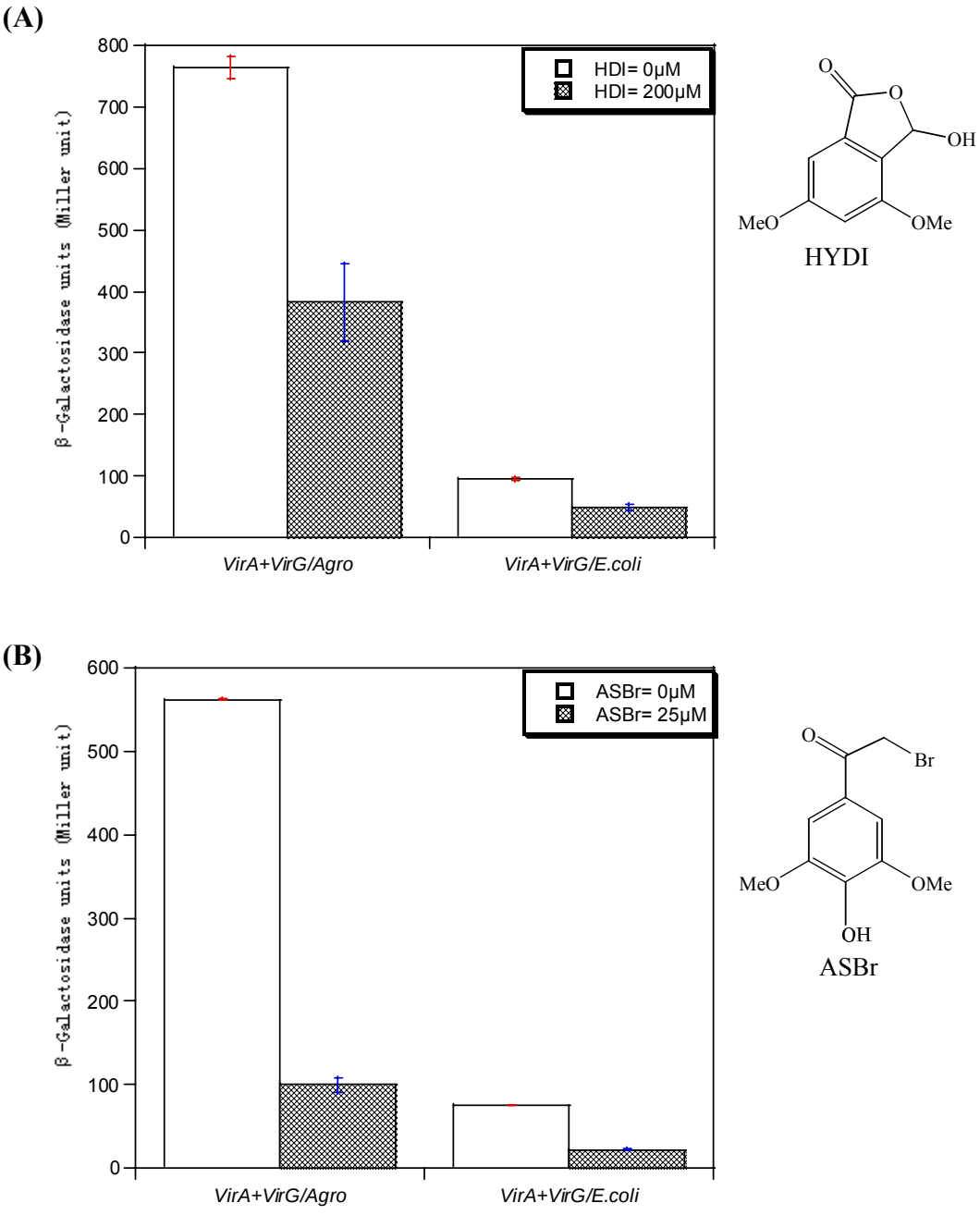
ASBr and HDI have been developed as competitive inhibitors for AS induction and therefore serve as additional probes for signal perception. If the phenols binding site differ in *A. tumefaciens* and *E. coli*, the activation of the inhibiting way also differs (Maresh *et al*. 2006). Fig 2.4 shows ASBr and HDI both inhibit induction in *E. coli* and the degree of inhibition are similar in both organisms. These data is further consistent with that VirA serves as the direct signal sensing for phenolic compounds.

Discussion

Prokaryotes, fungi and plants sense and adjust the environmental fluctuation through two-component regulatory systems that contains HK (histidine kinase) and RR (response regulator) domains. Environmental stimuli can be detected directly or indirectly by the N-terminal sensing domain of the HK. Until now, although a number of small molecules have been identified for initiating histidine kinases, in most cases, the specific stimuli and the mechanism of sensing domains remain unclear (Bader *et al*. 2005).

Phenolic compounds play the critical role for activation of VirA/VirG two-component system while monosaccharide and acidic pH act synergistically to enhance both phenol sensitivity and the response magnitude. Evidence has explicated that the VirA periplasmic domain senses monosaccharide and acidic pH through the protein ChvE (Peng *et al*. 1998, Gao *et al* 2005). However, this is the same exception as seen in

Figure 2.4 Inhibition of *vir* gene induction in both *A. tumefaciens* and *E. coli.* (A) *vir* gene induction in *A. tumefaciens* A136 containing pFQ25 and *E. coli* DH5α containing pFQ25 and pPS1.3 in 100μM AS for 16 hrs. (A) with and without 25 μM ASBr. (B) with and without 200μM HDI.



VirA, one of the most extensively studied TCS HK, the perception of the critical phenol is not known. Of the two possibilities: (1) multiple receptors exist in *A. tumefaciens*, (2) VirA is the direct structurally diverse sensor, my study argues strongly for VirA serving as the perception event. In the heterologous host *E. coli*, the inducer AS and inhibitors (HDI and ASBr) have been used and found out to function similarly in both *E. coli* and *A. tumefaciens*. To detect the diverse phenols, VirA is likely to have a generic detection system such as the proton transfer model, which functions independent of the structures of phenolic compounds (Lee *et al*. 1993). However, even when VirA was placed under the stronger P_{N25} promoter and cytoplasm VirA truncations were constructed to avoid mislocalization of VirA, the *vir* gene induction is still much lower in *E. coli*. The reason may be that *rpoA* from *E. coli* competes with r*poA* from *A. tumefaciens* for recognizing *vir* gene promoters. To resolve this problem, *E. coli rpoA* could be engineered to activate *virB::lacZ* expression. However, for these studying, I have focused on further dissecting the VirA receptor to characterize the mechanism of phenol perception and regulation.

CHAPTER 3

Role of the Interaction between the Linker and the Receiver in the Phenolic Activation of *A. tumefaciens* Virulence Genes

Introduction

Since I have further supported VirA's direct role in phenol perception, it is necessary to understand how VirA perceives, integrates and translates this signal information. VirA, as well as some other histidine kinases, have accumulated multiple input domains, implicating sophisticated signal integration mechanisms within a single protein. VirA activates the expression of the virulence genes (*vir*) in response to host-derived signals at plant wound sites. The transfer of oncogenic DNA into host cells (Tzfira *et al.* 2002; Winans *et al.* 1994; Zhu *et al.* 2000) requires phenols (e.g. acetosyringone [AS]) with monosaccharide (including glucose) and acidic pH (appropriately pH 4.8 to 5.5) to act synergistically as signals (Winans *et al.* 1994). Aside from a conserved kinase domain (K) for phospho-transfer to VirG, VirA contains at least two signal-input domains, the periplasmic domain (P) responsible for sensing low pH/monosaccharide and the linker domain (L) involved in phenol perception (Chang *et al.* 1992; Gao *et al.* 2005). An additional C-terminal receiver domain (R) also appears critical for regulation (Chang *et al.* 1996; Jin *et al.* 1990; Mukhopadhyay *et al.* 2004).

The presence of the receiver domain defines VirA as a hybrid kinase. The function of VirA's receiver appears to differ from those of some other hybrid kinases. First, the receiver domain was described as an inhibitory element since its removal of the receiver

allowed *vir* gene expression in the absence of a phenolic inducer, provided sugar and acidic pH were present (Chang *et al*. 1992; Chang *et al*. 1996; Brencic *et al*. 2004; Gao *et al*. 2005). Second, D766 in the receiver domain, the residue analogous to Asp52 on the VirG receiver domain, was not phosphorylated (Mukhopadyhay *et al*. 2004). It was previously reported that overexpressed VirA(712-829)(D766N) strongly decreased the basal activity of VirAΔR (Chang *et al.* 1996). In other words, to repress VirA activation, the receiver must interact with the other domains of VirA to repress either VirA autophosphorylation or phospho-transfer from VirA to VirG; however, this interaction has not yet been defined.

To better define the interaction between the receiver and VirA's other domains, null mutations in the VirA receiver domain were selected through library screen. Compensating suppressors of the null mutants were further identified both in the linker and kinase domains through a second library screen. Remarkably, VirA alleles such as LKR(Y293T/V765D) significantly enhance both the sensitivity and maximal response to AS induction, demonstrating a direct interaction between the linker and the receiver domain. In vitro cross-linking results further support the existence of this interaction. It is proposed that the receiver domain represses VirA activation through the interaction between the linker and the receiver, and with phenols, the linker disassociates from the receiver to relieve this repression. Within the two-component systems, this is the first proof that a linker domain interacts directly with a receiver.

Material and methods

Bacterial Strains, Plasmids and Reagents. The bacterial strains and plasmids used in

this study are listed in Table 3.1. *E. coli* strains XL1-Blue, XL10-Gold and DH5α were used for routine cloning. Acetosyringone (AS) used for *vir* induction assays was purchased from Sigma-Aldrich Corp. The enzymes for DNA manipulation were purchased from either New England Biolab or Promega unless specified.

Table 3.1 Bacterial strains and plasmids used in Chapter 3

Strains /plasmids	Relevant characteristics	Reference
E. coli.		
XL1-Blue	*recA1 endA1 gyrA96 thi¯1 hsdR17 supE44 relA1 lac[F' proAB lacI^{q}Z M15 Tn 10* (Tc^r)*]*	Stratagene
XL10-Gold	Tet^r *(mcrA)183 (mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F' *proAB lacI^{q}Z M15 Tn10* (Tet^r) Amy Cam^r]^a	Stratagene
DH5α	*recA1 endA1 hsdR17 supE44 gyrA96 relA1Δ(lacZYA-argF) U169 (ϕ80dlacZΔM15)*	Invitrogen
A .tumefaciens		
A136	Strain C58 cured of pTi plasmid	(Watson *et al*. 1975)
A348-3	C58 background with pTiA6NC, *virA*::*kan^r*	(Lee *et al*. 1992)
Plasmids		

Strains /plasmids	Relevant characteristics	Reference
pSW209Ω	IncP plasmid carrying P*virB-lacZ* fusion, *Spec*^{r}	(Campbell *et al*. 2000)
pQF114	P_{N25}:LKR(285-829)(V765D) in pJZ6	This chapter
pQF113	P_{N25}:LKR(285-829)(L770P) in pJZ6	This chapter
pQF367	P_{N25}:LKR(285-829) in pJZ6	This chapter
pQF127	P_{N25}:LKR(285-829)(T367A) in pJZ6	This chapter
pQF144	P_{N25}:LKR(285-829)(T367A, L770P) in pJZ6	This chapter
pQF298	P_{N25}:LKR(285-829)(T367A, V765D) in pJZ6	This chapter
pQF299	P_{N25}:LKR(285-829)(T367A, D766N) in pJZ6	This chapter
pQF192	P_{N25}:LKR(285-829)(M515K) in pJZ6	This chapter
pQF193	P_{N25}:LKR(285-829)(M515K, L770P) in pJZ6	This chapter
pQF340	P_{N25}:LKR(285-829)(M515K, D766N) in pJZ6	This chapter
pQF341	P_{N25}:LKR(285-829)(M515K,V765D) in pJZ6	This chapter
pQF203	P_{N25}:LKR(285-829)(K368I) in pJZ6	This chapter
pQF202	P_{N25}:LKR(285-829)(K368I, D776N) in pJZ6	This chapter
pQF336	P_{N25}:LKR(285-829)(K368I,L770P) in pJZ6	This chapter
pQF337	P_{N25}:LKR(285-829)(K368I,V765D) in pJZ6	This chapter
pQF217	P_{N25}:LKR(285-829)(G470S) in pJZ6	This chapter

Strains /plasmids	Relevant characteristics	Reference
pQF216	P_{N25}:LKR(285-829)(G470S, D766N) in pJZ6	This chapter
pQF338	P_{N25}:LKR(285-829)(G470S,L770P) in pJZ6	This chapter
pQF339	P_{N25}:LKR(285-829)(G470S,V765D) in pJZ6	This chapter
pQF190	P_{N25}:LKR(285-829)(Y293F) in pJZ6	This chapter
pQF358	P_{N25}:LKR(285-829)(Y293F, V765D) in pJZ6	This chapter
pQF356	P_{N25}:LKR(285-829)(Y293F, D766N) in pJZ6	This chapter
pQF357	P_{N25}:LKR(285-829)(Y293F, L770P) in pJZ6	This chapter
pQF188	P_{N25}:LKR(285-829)(H485Y) in pJZ6	This chapter
pQF189	P_{N25}:LKR(285-829)(H485Y, V765D) in pJZ6	This chapter
pQF309	P_{N25}:LKR(285-829)(H485Y, D766N) in pJZ6	This chapter
pQF310	P_{N25}:LKR(285-829)(H485Y,L770P) in pJZ6	This chapter
pQF333	P_{N25}:LKR(285-829)(Y293L) in pJZ6	This chapter
pQF353	P_{N25}:LKR(285-829)(Y293L, V765D) in pJZ6	This chapter
pQF294	P_{N25}:LKR(285-829)(Y293E) in pJZ6	This chapter
pQF307	P_{N25}:LKR(285-829)(Y293E, V765D) in pJZ6	This chapter
pQF334	P_{N25}:LKR(285-829)(Y293H) in pJZ6	This chapter
pQF355	P_{N25}:LKR(285-829)(Y293H, V765D) in pJZ6	This chapter
pQF117	P_{N25}: R(711-829)-flag in pJZ6	This chapter

Strains /plasmids	Relevant characteristics	Reference
pYW15b	P_{N25}:Broad-host-range expression vector, IncW, Ap^r	(Wang *et al*. 2000)
pRG15	P_{N25}:His-L(285-471) in pYW15b	(Rong 2004)
pQF295	P_{N25}:LKR(285-829)(Y293S) in pJZ6	This chapter
pQF267	P_{N25}:LKR(285-829)(Y293P) in pJZ6	This chapter
pQF282	P_{N25}:LKR(285-829)(Y293T) in pJZ6	This chapter
pQF335	P_{N25}:LKR(285-829)(Y293A) in pJZ6	This chapter

Table 3.2 Primers used in Chapter 3.

Primer Name	Sequence	Characteristics
LKR285	CG *GGATCC* GAT TGG TTA GCG CGG CGT	BamH I & Sense
LKRA	GC *GGTACC* GCA ACT CTA CGT CTT GAT	Kpn I & antiSense
D766NF	GTC ATG GTC AAC CAA GCG	D766N & Sense
D766NR	CGC TTG GTT GAC CAT GAC	D766N & antiSense
V765DF	CTG GTC ATG GAC GAC CAA GCG	V765D & Sense
V765DR	CGC TTG GTC GTC CAT GAC CAG	V765D & antiSense

Primer Name	Sequence	Characteristics
L770PF	CAA GCG TCT CCT CCT GAA GAT	L770P & Sense
L770PR	ATC TTC AGG AGG AGA CGC TTG	L770P & antiSense
K368IF	CGT ACC ATA GCG GAC GAA	K368I & Sense
K368IR	TTC GTC CGC TAT GGT ACG	K368I & antiSense
G470SF	ACA CTT GCC AGC GGA ATA	G470S & Sense
G470SR	TAT TCC GCT GGC AAG TGT	G470S & antiSense
Y293FF	CG *GGATCC* GAT TGG TTA GCG CGG CGT TTA GAT TTC	BamH I & Sense
H485YF	CTC GGG TAC GCA GAA TTA	H485Y & Sense
H485YR	TAA TTC TGC GTA CCC GAG	H485Y & antiSense
T367AF	GTC TCT CGT GCC AAA GCG	T367A & Sense
T367AR	CGC TTT GGC ACG AGA GAC	T367A & antiSense
M515KF	GAC AGA GCC AAG CTC ATT	M515K & Sense
M515KR	AAT GAG CTT GGC TCT GTC	M515K & antiSense

Primer Name	Sequence	Characteristics
A6RRXBA I	GC *TCTAGA* CTA GAT CTT ATC GTC GTC ATC CTT GTA ATC CGT CTT GAT TTT GGT	Xba I & antiSense with flag
YL005	GCT *GAGCTC* GGA AAC GGG GAG ATT GTG GC	Sac I & Sense
Y293EF	CG *GGATCC* GAT TGG TTA GCG CGG CGT TTA GAT GAG GAA	BamH I & Sense
Y293LF	CG *GGATCC* GAT TGG TTA GCG CGG CGT TTA GAT CTC	BamH I & Sense
Y293SF	CG *GGATCC* GAT TGG TTA GCG CGG CGT TTA GAT TCC GAA	BamH I & Sense
Y293PF	CG *GGATCC* GAT TGG TTA GCG CGG CGT TTA GAT CCC GAA	BamH I & Sense
Y293TF	CG *GGATCC* GAT TGG TTA GCG CGG CGT TTA GAT ACC GAA	BamH I & Sense
Y293AF	CG *GGATCC* GAT TGG TTA GCG CGG CGT TTA GAT GCC	BamH I & Sense
2626F	GCT ATC GCC CTC GAC CTT GCC AAG GC	Position in VirA & Sense
2790R	CCG CCG GCA AGT GTA CCA ACT GCC TCA	Position in VirA & antiSense
3133F	GAA TGC TTC CCA AGC CAT GAC TGC AAA TGG	Position in VirA & Sense
3580R	CCG ACC GGC TCA TAT CCT AGA GCG GCG	Position in VirA & antiSense

Plasmid constructions. The plasmids used in this study are listed in Table 3.1. The coliphage T5 promoter (P_{N25}) was used to drive the expression of *virG* independent of environmental factors like pH and phosphate concentration (Wang *et al*. 2000). The Nco I fragment containing P_{N25}-6xHis-*virG* from pYW47 was blunt-ended with Klenow fragment and ligated with pJZ4 that was treated with Kpn I and Klenow fragment, resulting in pRG109 (Gao *et al*. 2005). To give plasmid pQF117, The fragment R(712-829) was amplified with primers A6RRXBA I and YL005 and pYW48 as template. The fragment was digested with Sac I and Kpn I and inserted into pJZ6. To make the plasmid pQF367, PCR used primers LKR285 and LKRA and pYW48 as template. The PCR product was digested with BamH I and Acc65 I and then inserted into pJZ6. Site-specific mutagenesis was performed using the recombinant PCR with primers shown in Table 3.2. The primary PCRs used pYW48 as template with primers: (1) LKR285 and the antisense primers containing the mutation; (2) the sense primers containing the mutation and LKRA. Primary PCR products 1 and 2 were gel-purified and mixed as templates for the secondary PCR with LKR285 and LKRA as primers. The secondary PCR products were gel-purified and digested with BamH I and Acc65 I. The digestion products were inserted into vector pJZ6 at the same restriction cutting sites to create plasmids with mutations listed in the Table 3.1. Primers used in recombinant PCR were listed in the Table 3.2. For plasmids containing double mutations, the single mutation was obtained as above and the double mutations were created using these plasmids as template in site-specific mutagenesis. All mutations were confirmed by DNA sequencing using primers 2626F, 2790R, 3133F or 3580R.

Library screen for null VirA alleles. LKR alleles following random mutagenesis in the

VirA receiver (712-829) (GeneMorph® II Random Mutagenesis kit) were inserted into pJZ6 and transformed into A136/pRG109. The transformants were plated on I.M. with X-gal and 100μM AS. White colonies were picked and confirmed to be inactive with liquid assays. The colonies showing null phenotype were used for colony PCR and the PCR products were sequenced.

Recovering suppressors of the null mutants. The fragment of LKR(285-829) with BamH I and Sac I cutting sites were used as template for random mutagenesis PCR (GeneMorph® II Random Mutagenesis kit). The amplified products were digested with BamH I and Sac I to replace the same fragment of LKR(285-829)(V765D, D766N or L770P) and transformed into A136/pRG109. The transformants were plated on I.M. with X-gal and 100μM AS. Blue colonies were picked and confirmed to be active with liquid assays. The alleles from these colonies were amplified using colony PCR and sequenced.

Immunoblot analysis. *A. tumefaciens* cells were grown overnight in 50 mL LB with the appropriate antibiotics. The bacteria were pelleted and washed with phosphate-buffered saline (PBS), and lysed by brief sonication (Amp: 25-30%, time: 30 sec, pulse: on 1.0 sec and off 3.0 sec) on ice. Clarified lysates through centrifugation were analyzed by SDS-PAGE in 10% polyacrylamide gels (Invitrogen) followed by electro-blotting onto nitrocellulose membranes using the mini trans-blot transfer apparatus (Bio-Rad). To detect VirA truncation alleles, the membranes were blocked with 3% BSA in Tris-buffered saline (TBS) and probed with anti-VirA polyclonal Antibody (Binns *et al*, unpublished) at 1:200 dilution. Visualization was achieved using goat anti-rabbit antibody conjugated to alkaline phosphatase (Amersham) at 1:1000 dilution, followed by 1-Step NBT/BCIP (Pierce) developing.

β-Galactosidase assays for *vir* gene induction. pRG109 carrying the β-galactosidase reporter P_{virB}-*lacZ* and P_{N25}-*virG* or pSW209Ω carrying P_{virB}-*lacZ* and P_{vir}-*virG* were used to assay *vir* gene expression in *Agrobacterium* strains A136 or A348-3. Cells were grown in 20 mL of LB medium to an OD_{600} 0.4-0.6 in the presence of the appropriate antibiotics. The cells were pelleted by centrifugation for 10 min at 7000 x *g* at 4°C and washed with PBS. The pellet was diluted to an OD_{600} of ~ 0.1 into tubes containing a total of 1 mL of induction media (I.M.) with a fixed concentration of AS (Cangelosi *et al*. 1991) and cultured at 28°C with shaking at 225 rpm for 15 hrs. β-galactosidase activity was determined as described (Miller 1972). Optical densities were determined at 600 nm and 415 nm using the EL800 microplate reader (BIO-TEK Instruments).

***In vitro* crosslinking procedures**. Linker and receiver domains were expressed in *E. coli* at 25°C, purified with Ni-TNA column separately and reacted with crosslinker reagent dimethyl suberimidate (DMS) at the ratio 1:10. The reaction was incubated at RT for 1 hr, and quenched with 20 μL of quenching buffer (2.5 M Tris·HCl, pH 7.0) for 15 minutes. 2xSDS sample buffer (Invitrogen) was added to the reaction mixture and boiled for 5 minutes prior to the SDS-PAGE analysis. Samples were separated using SDS-PAGE and visualized through Western blot using the anti-PentaHis antibody (Invitrogen).

Results

Null mutants in the receiver domain are located proximal to D766N

Null mutations (V765D, D766N and L770P) of VirALKR(285-829) alleles were

obtained in the receiver domain by library screen described in the method about Library screen for null VirA alleles. According to the sequence alignment of the receiver domain from five VirA proteins (Fig 3.1), VirA receiver domain consists of a typical α and β architecture with five α-helix and five β-sheets. V765 and D766 are located in β3 while L770 is in the loop connecting β3 and α3. Each of these residues resides within or close to the predicted active site of typical response regulators.

Suppressor mutations of the LKR(285-829)(V765D, D766N and L770P) phenotypes

Library screen in the linker and kinase domains was utilized to select for suppressors of the null mutants described above. As shown in Fig 3.2, the linker and part of kinase domains were amplified via random mutagenesis PCR and swapped in wild type LKR using BamH I and Sac I cutting sites in LKR(285-829)(D766N). The ligation mixture of LKR(285-829)(D766N) with random mutations was transformed in *Agrobacterium* and plated in I.M. with 100 μM AS and X-gal. Blue colonies (0.2% of the whole colonies) were selected and sequenced. Two mutations, K368I and G470S, one in the linker domain and one in the kinase domain were selected for further study. As shown in Fig 3.3, the LKR(285-829)(K368I) or LKR(285-829)(K368I/D766N) alleles in 200μM AS appear less active than wild type LKR(285-829), but both have higher P_{virB}-*lacZ* expression than the null mutant LKR(285-829)(D766N). To determine whether these mutations are specific to the null D766N substituent, plasmids containing V765D and L770P with K368I and G470S were constructed and transformed into *Agrobacterium*. As shown in Fig 3.3, alleles (K368I/V765D, K368I/L770P and G470S/V765D) were all activated with 200μM AS, establishing that K368I and G470S suppress all three null

Figure 3.1 Sequence alignments of receiver domains. The secondary structure prediction employed CLUSTAL W software and five receiver amino acid sequences from VirA strains pTiA6, pTi15955, pTiC58, RU12 and pTiAg162. Asterisk indicates identical amino acids in different VirA proteins. Two-dots means conserved amino acid. One-dot means less conserved. Residues V765, D766 and L770 are indicated in red.

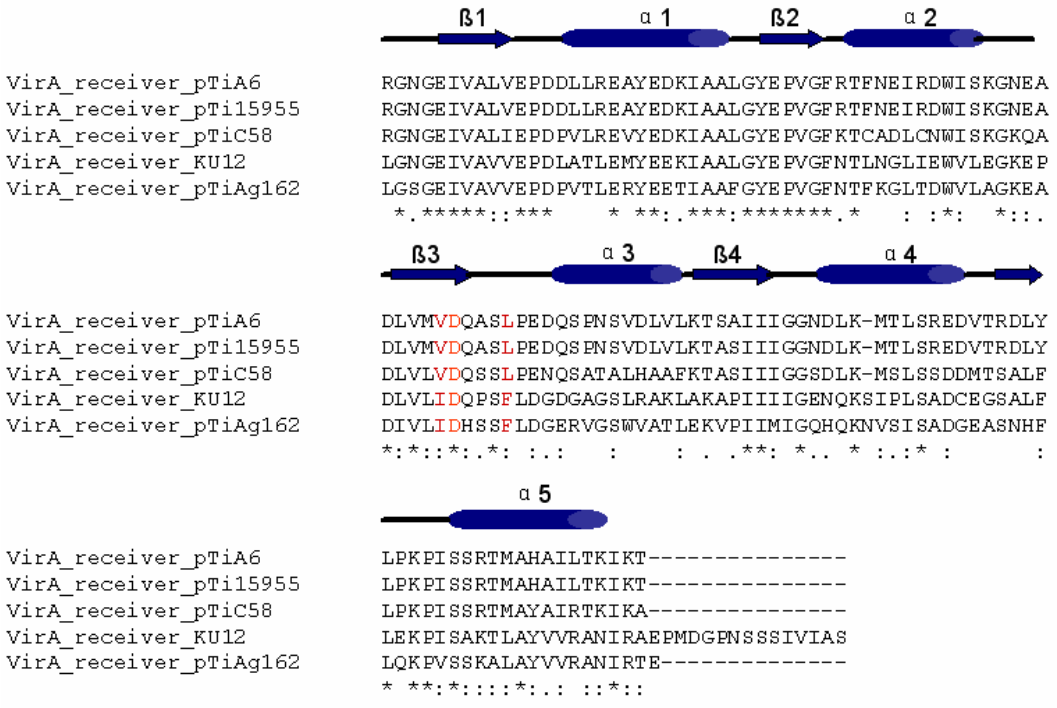


Figure 3.2 Library screen scheme for rescue of LKR(D766N) repression. The fragment with BamH I and Sac I cutting sites of LKR(285-829)(D766N) was amplified with random mutagenesis PCR and transformed into *Agrobacterium* strain including P_{N25} driven VirG and reporter system *virB:lacZ*. The transformants was plated on IM with X-gal and 100 μM AS. Blue colonies were picked and sequenced. Orange, linker domain; purple, kinase domain; blue, receiver domain.

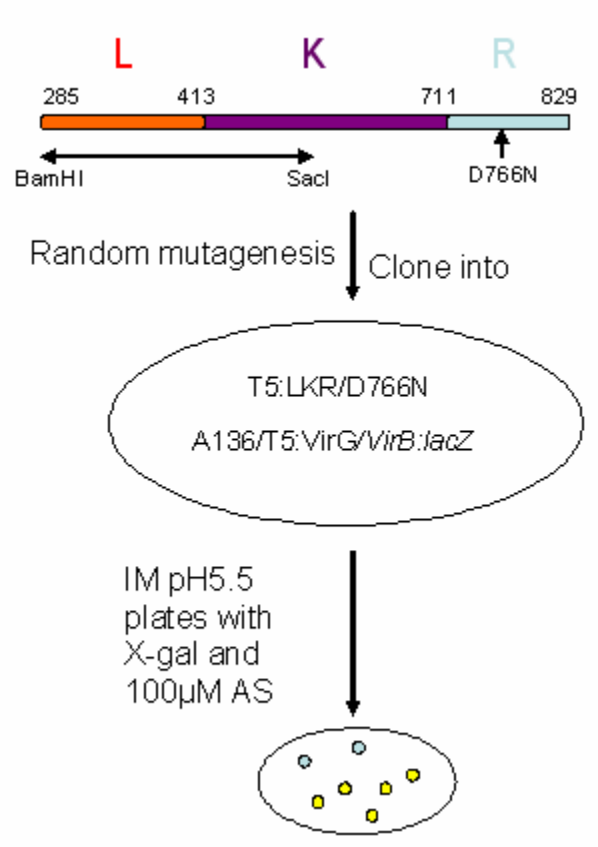
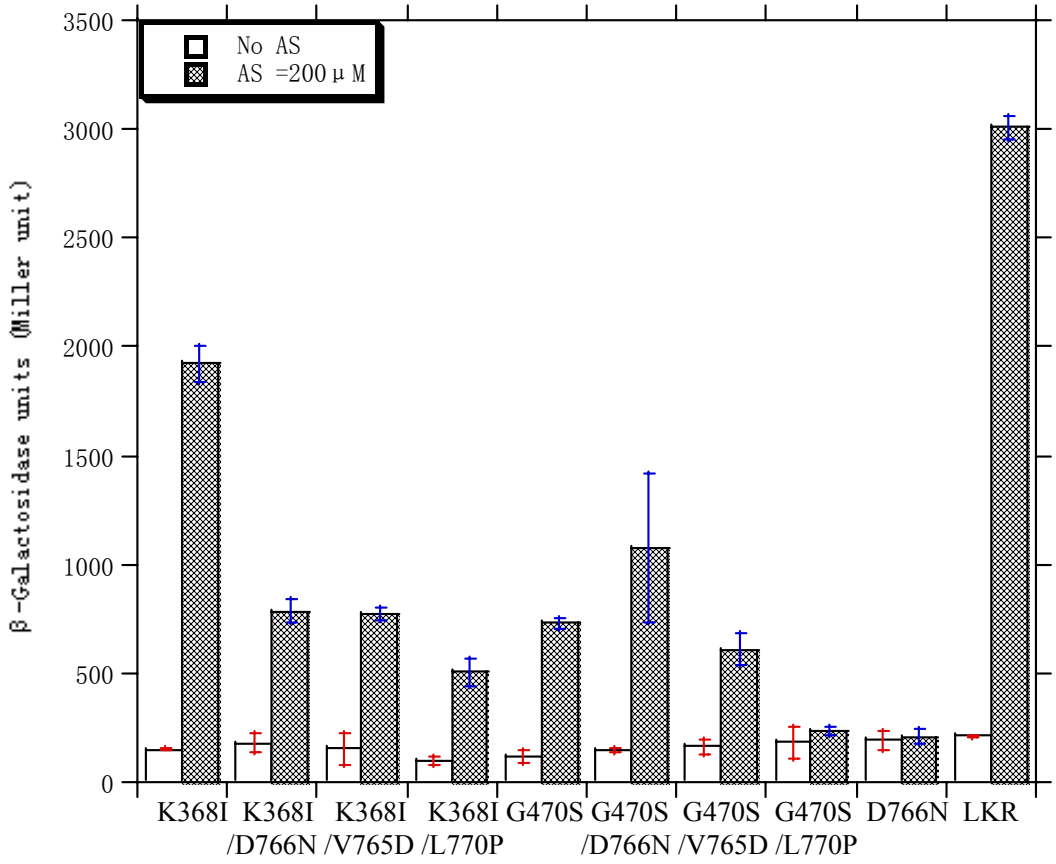


Figure 3.3 Suppressor alleles of LKR(285-829)(D766N). *A. tumefaciens* (A136/pRG109) carrying indicated plasmids pQF203, pQF202, pQF336, pQF337, pQF217, pQF216, pQF338, pQF339 and pQF367 were cultured in induction medium with 200μM AS and assayed for the *vir* gene expression as described in the method.



substituents in the receiver. Western blot analysis with VirA antibody (Fig 3.6) shows protein expression levels even though the background levels with this antibody are too high for quantitative assessment.

Mutations in the linker and kinase domains were also selected for overcoming the suppression of LKR(285-829)(V765D) and LKR(285-829)(L770P). Two alleles, Y293F and H485Y, were found in the linker and kinase domains respectively for LKR(285-829)(V765D)(Fig 3.4) while T367A and M515K were found for LKR(285-829)(L770P) (Fig 3.5). Again, western blot with the VirA antibody suggests that all constructs except LKR(285-829)(M515K) have similar protein expression (Fig 3.6) indicating that the substituents, not protein expression, lead to the activation difference among these constructs. Surprisingly, Y293F in the linker domain suppresses the null phenotype of V765D, L770P and D766N dramatically. As shown in Fig 3.4 and in Fig 3.7, LKR(285-829)(Y293F) is most remarkable. The sensitivity to AS is dramatically shifted and LKR(285-829)(Y293F/V765D) is both more sensitive and significantly more active than wild type LKR(285-829) at all concentrations of AS. The *vir* induction assay was also investigated in the strains carrying native P_{virG} promoter to test whether expression of VirG affects the function that all constructs have similar of these alleles or not. As shown in Fig 3.8, LKR(285-829)(Y293F/V765D) is still significantly more active than wild type LKR(285-829) when VirG is driven by its native promoter. These results might suggest that the linker domain interacts with the receiver domain to relieve repression of VirA activation. To test whether the linker interacts with receiver domain, both linker and receiver with His-tags were purified with Ni-TNA columns, and the purified proteins were incubated with crosslinking reagent DMS (DMS structure shown in Fig 3.9A).DMS

Figure 3.4 Suppressor alleles of LKR(285-829)(V765D). *A. tumefaciens* (A136/pRG109) carrying indicated plasmids pQF190, pQF358, pQF356, pQF357, pQF188, pQF189, pQF309, pQF310, pQF367 and pQF114 were cultured in I.M. supplemented with 1% glycerol and 200 µM AS and assayed for P_{virB}-*lacZ* expression.

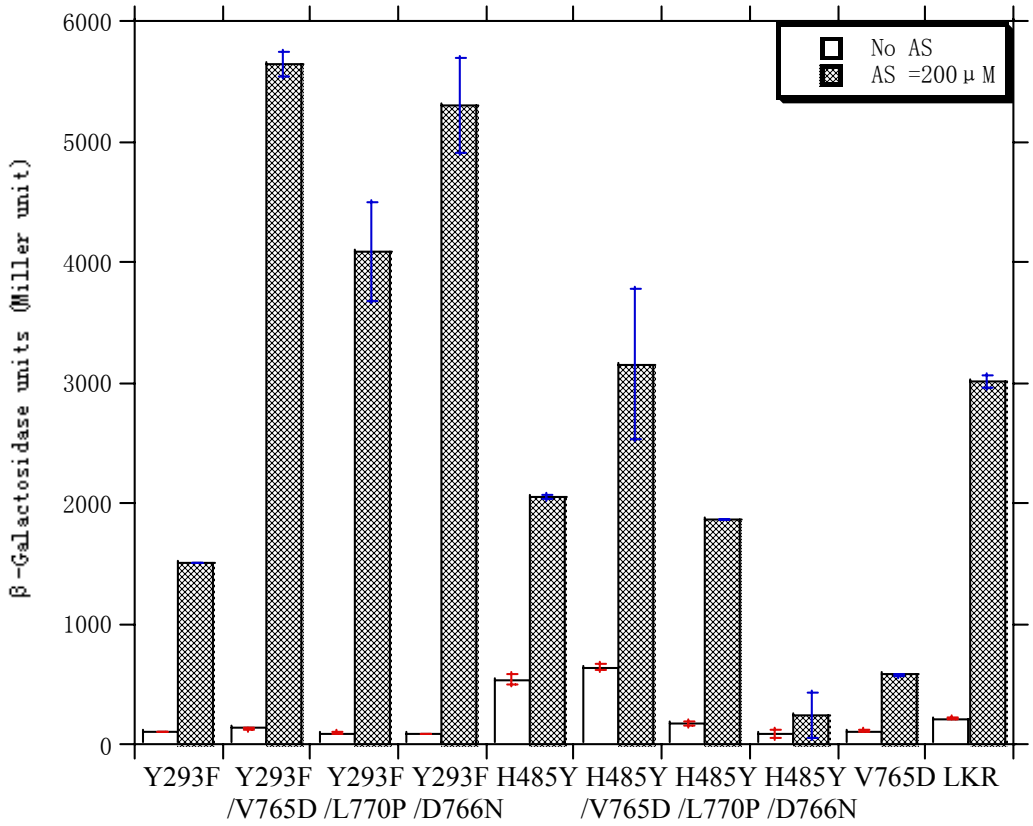


Figure 3.5 Suppressor alleles of LKR(285-829)(L770P). *A. tumefaciens* (A136/pRG109) carrying indicated plasmids pQF127, pQF144, pQF298, pQF192, pQF192, pQF193, pQF340, pQF341 and pQF367 were cultured in I.M. supplemented with 1% glycerol and 200μM AS for P_{virB}-*lacZ* expression.

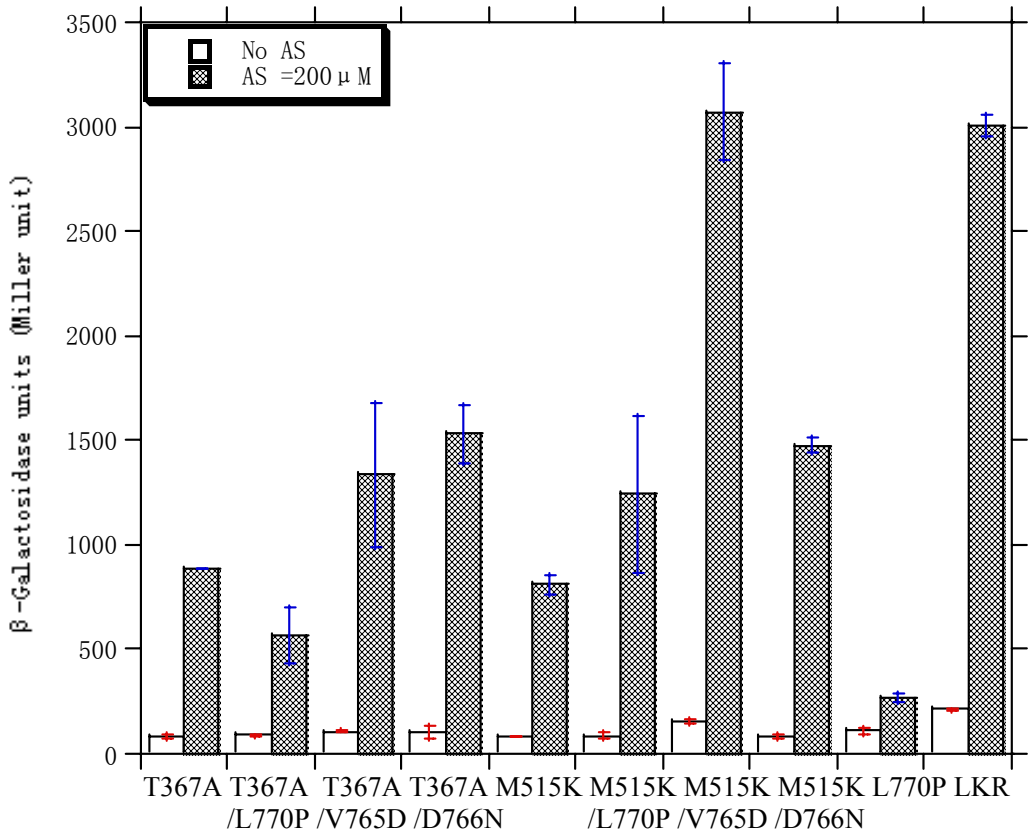


Figure 3.6 Western blot analysis of protein expression of all constructs. Cell lysates were analyzed by 10% SDS-PAGE and detected by anti-VirA polyclonal antibodies.

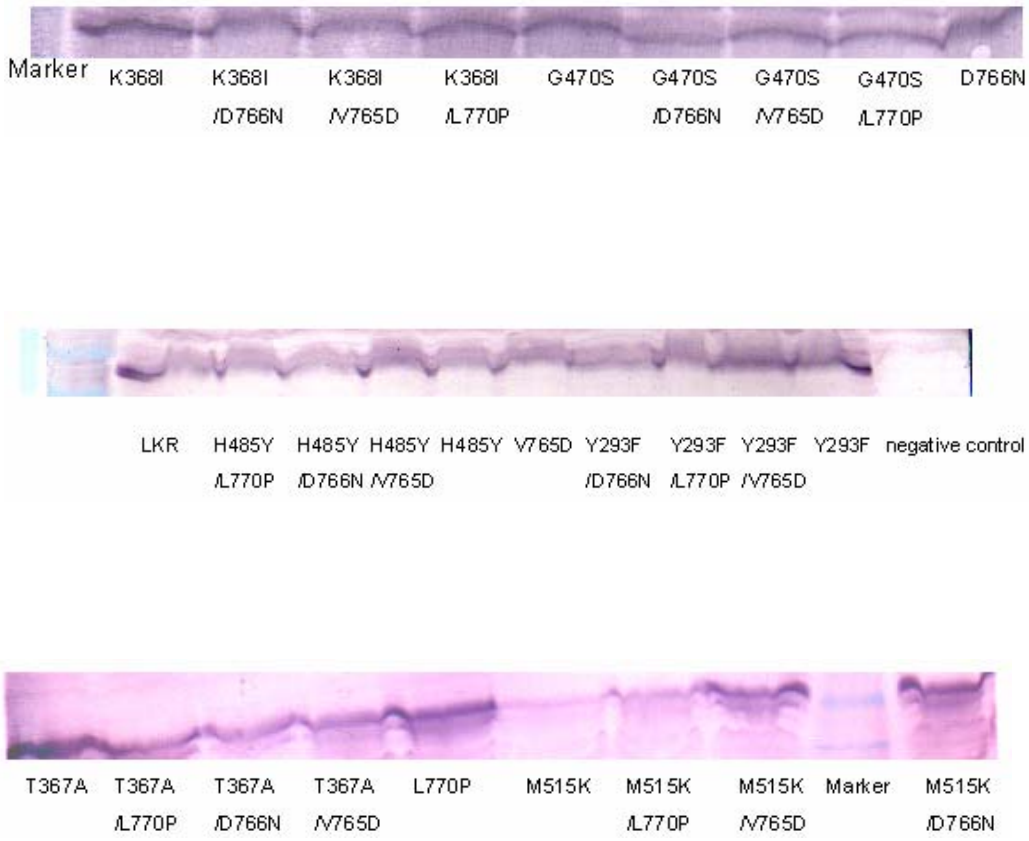


Fig 3.7 AS dose response of LKR(285-829), LKR(285-829)(Y293F/V765D), and LKR(285-829)(Y293F). *A. tumefaciens* (A136/pRG109) carrying indicated plasmids pQF190, pQF358, pQF367 and pQF114 were cultured in I.M. supplemented with 1% glycerol and the indicated concentrations of AS for P_{virB}-*lacZ* expression.

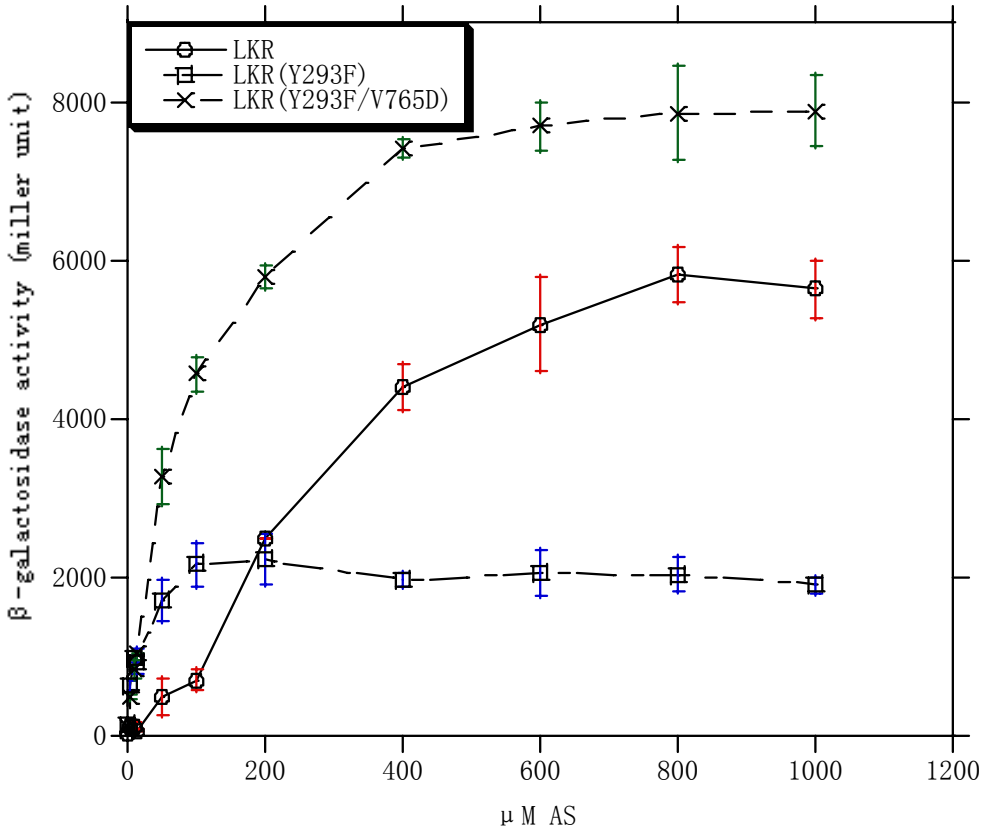
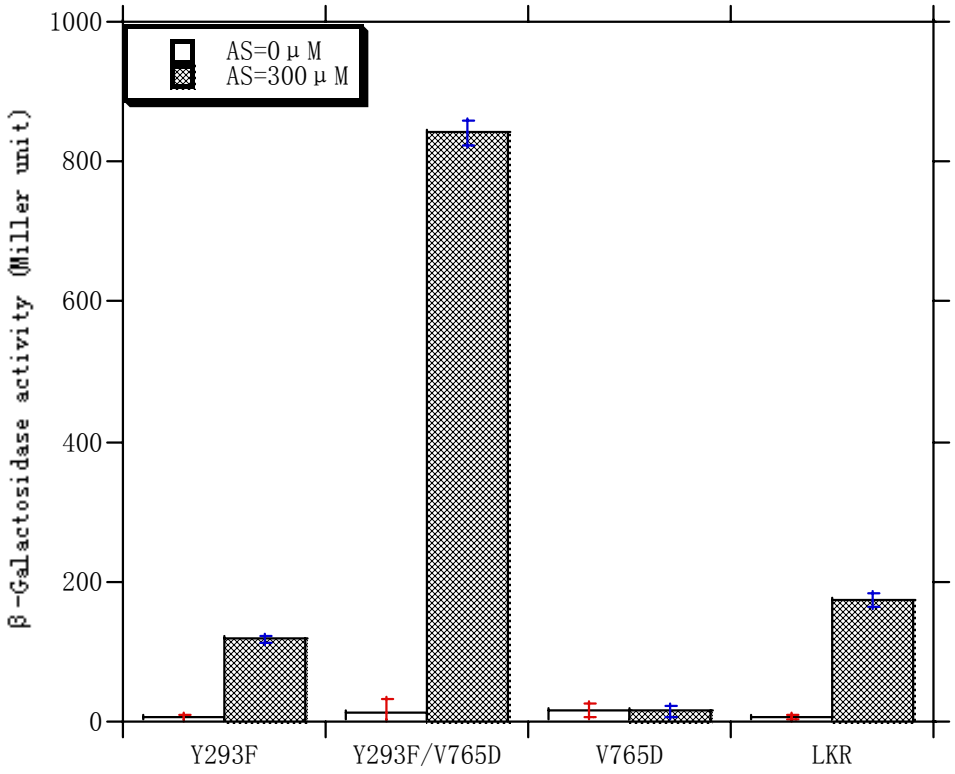


Fig 3.8 Activation of LKR(285-829) and its alleles in a Pvir:virG background. *A. tumefaciens* (A348-3/pSW209Ω) carrying P_{vir}:*virG* and the indicated plasmids pQF190, pQF358, pQF367 and pQF114 were cultured in I.M. supplemented with 1% glycerol and 300 μM AS for P_{virB}-*lacZ* expression.



covalently links adjacent primary amines to form bis-imidoesters. As shown in Fig 3.9B, a protein band with the size of L+R (around 36kDa) was observed following DMS treatment. As a control, linker and receiver fragments were incubated without DMS under the same condition and no protein band corresponding to L+R was observed. To test whether the interaction between linker and receiver was specific, linker and BSA were incubated with DMS and no protein band with a similar size as L+BSA (around 120kDa) was found (data not shown). Taken together, the cross-linking experiments provide direct physical evidence that the linker interacts with the receiver which further supports the genetic results.

Requirements at aa293

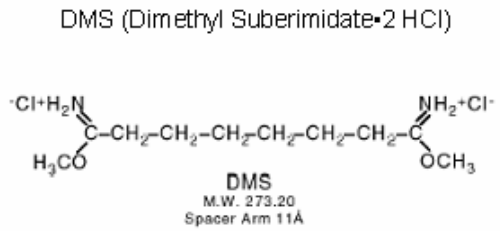
The role at aa293 seemed remarkable which led to a survey by saturation mutagenesis. Indeed, substituents at this position gave quite different phenotypes as shown in Fig 3.10. Aromatic residues seemed to be generally required for activity. However, Y293L appeared most active and Y293A was constitutive on. The other alleles were largely inactive at 300μM AS. These data clearly established aa293 is critical for VirA signal activation, although it is not clear whether it alters signal binding or signal transduction, nor it is clear how aromatic/hydrophobic residues affect the receiver domain.

Amino acid 293 and the receiver domain

Besides Y293F, Y293E, Y293L and Y293H were selected for constructing double mutant (Y293E, L, H/V765D). As shown in Fig 3.11, the information can be obtained as

Figure 3.9 In vitro cross-linking of linker and receiver domain. Purified linker and receiver were incubated with and without DMS as described in Materials and Methods. Crosslinked protein samples were separated by 10% Tris-Glycine gels and visualized by Western blot using anti-PentaHis antibodies. (A) The structure of dimethyl suberimidate-2HCl (DMS). (B) Western blot. (-): no DMS, (+) with DMS.

(A)



(B)

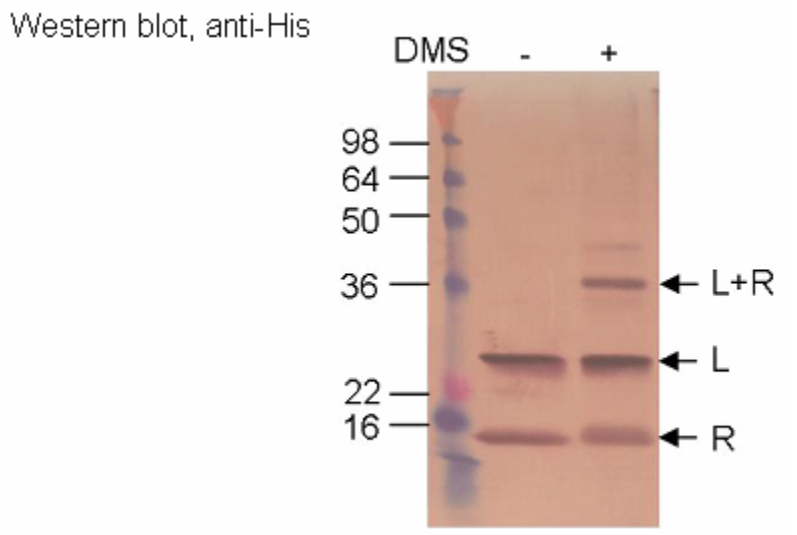


Figure 3.10 P_{virB}-*lacZ* expression by substituents at aa293. *A. tumefaciens* (A136/pRG109) carrying LKR(285-829)(Y293X) were cultured in I.M. supplemented with 1% glycerol and 300μM AS and assayed for P_{virB}-*lacZ* expression.

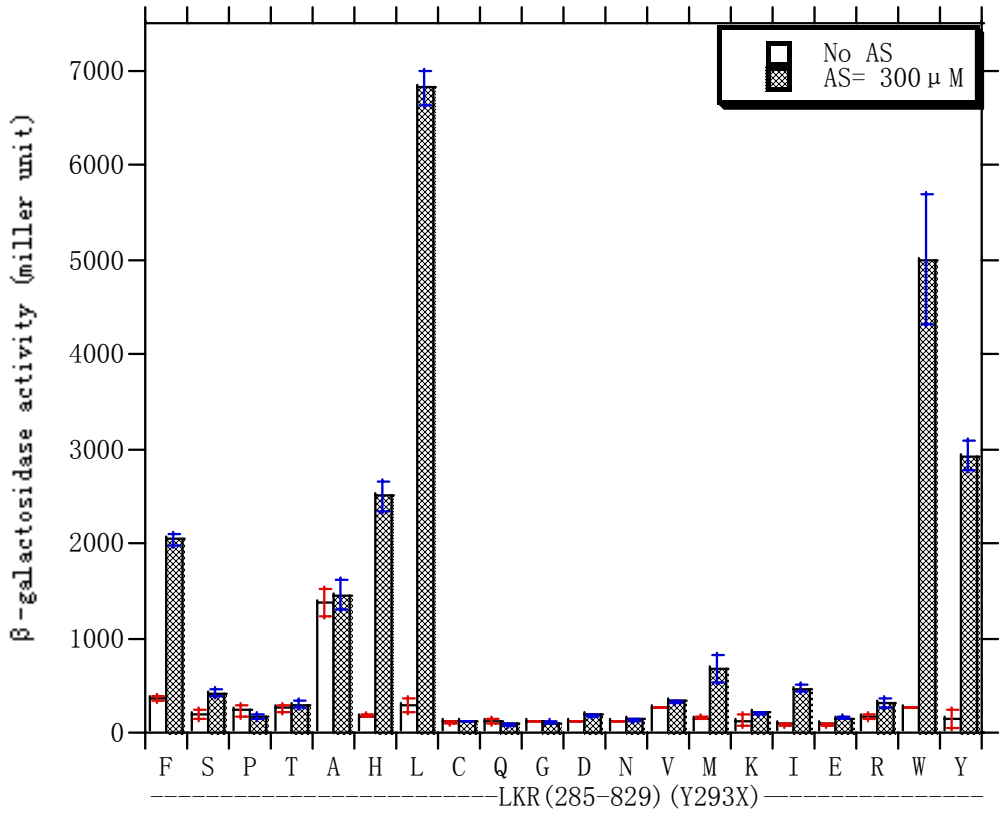
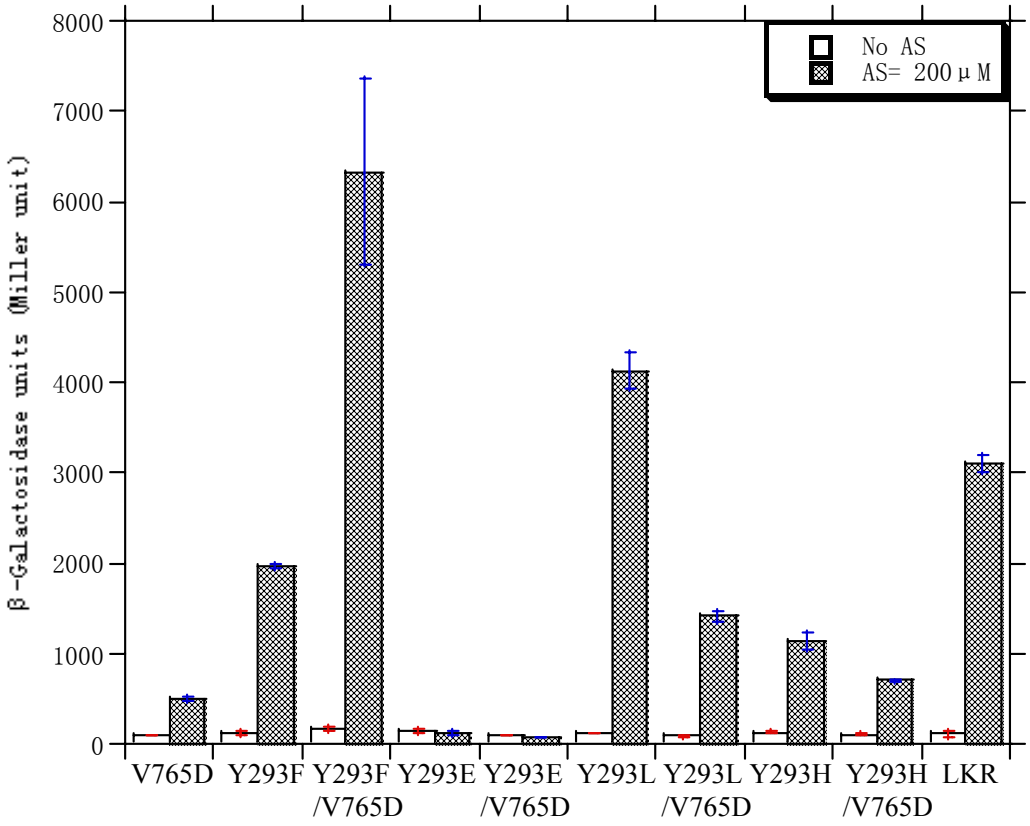


Figure 3.11 P_{virB}-*lacZ* expression by Y293F/E/L/H carrying V765D. *A. tumefaciens* A136/pRG109 carrying indicated plasmids pQF114, pQF190, pQF358, pQF294, pQF307, pQF333, pQF353, pQF334, pQF355 and pQF367 were cultured in I.M. supplemented with 1% glycerol and 300μM AS for P_{virB}-*lacZ* expression.



follows: (1) Y293E, an inactive substituent, remains inactive in double substituents. (2) While Y293L is the most active substituent, the addition of V765D lowers the activation. (3) Y293F/V765D is yet discovered as the most active construct. (4) Y293H shows very little change with the additional change in the receiver domain. All this information suggests there should have specific interaction between linker and receiver domain.

Discussion

In contrast to most two-component signal modules, VirA carries an additional domain at its C terminus whose amino acid sequence is homologous to the N-terminal of VirG. With constitutively expressed VirG, the VirA receiver acts as an inhibitory element. Chang *et al.* found that overexpressing VirA(712-829) or VirA(712-829)(D766N) strongly decreased the basal activity of VirAΔR as shown in Table 3.3, indicating that VirA(712-829)(D766N) strongly inhibits kinase activity and the inhibition does not require phosphorylation of this module. Rather, the inhibition by the receiver is more likely due to kinase-receiver interactions that mimic those of the wild-type protein (Chang *et al.* 1996). In the case of another two-component system, AmiC/AmiR, the crystal structure of AmiC-AmiR complex also identifies AmiR as a new and unique member of response regulator family of bacterial signal transduction proteins controlled by signal-regulated sequestration rather than phosphorylation (O'Hara *et al.* 1999). In the AmiC-AmiR complex, AmiC surface residues make a highly complementary interaction with the AmiR dimer. With acetamide binding to AmiC, the AmiC-AmiR association is

disrupted.

D766N gives a VirA null phenotype. Here two additional null mutations V765D and L770P) are discovered and both are close to the active site. Compensating alleles were found in the kinase domain, supporting the above hypothesis. Unexpectedly, compensating alleles were also found with substituents in the linker. The double substitution alleles e.g. LKR(285-829)(Y293F/V765D), are particularly noteworthy. The dramatic activity of Y293F/V765D implicates an interaction between the linker and the receiver. Physical evidence (crosslinking data) supports the existence of this interaction.

Recently, the structure of the complex ThkA/TrrA has been solved by a combination of X-ray crystallography and small-angle X-ray scattering. Those results found a PAS domain within the kinase ThkA that interacts with the response regulator TrrA (Fig 3.12), leading to the proposal that TrrA feedback regulates kinase activity (Yamada *et al.* 2006). Based on these results, I suggest that in addition to the interaction between the kinase and the receiver domain, there is an additional interaction between the linker and receiver domain. With signal induction, the linker relieves the inhibition by the receiver. Mutations such as V765D, D766N and L770P in the active sites of the receiver domain strengthen the interaction with the linker domain such that de-repression is not possible with signal induction. However, compensating substituents in the linker domain are able to weaken this interaction and relieve the inhibition. These data provide the first direct proof, both physical and genetic, that a linker domain can interact with a receiver domain in TCS signaling.

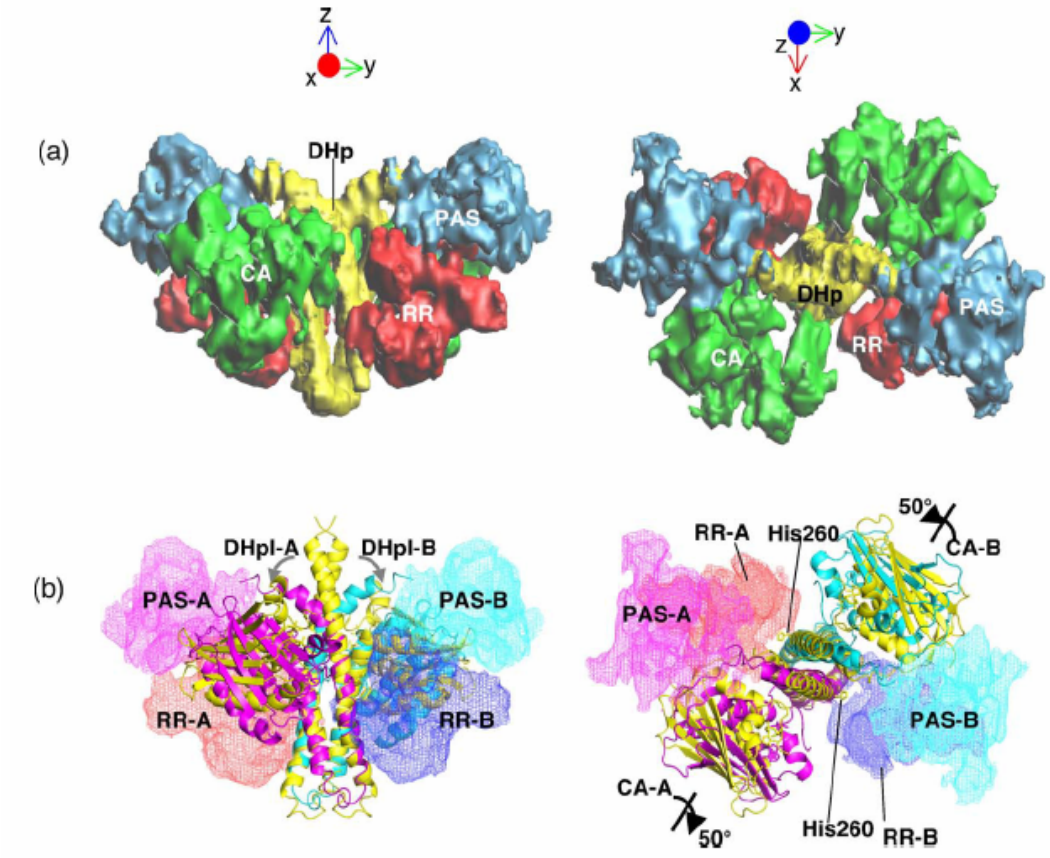
LKR(285-829)(Y293F/V765D) significantly enhances the sensitivity and maximal response to AS, and saturation mutagenesis at aa293 identified the importance of

hydrophobic and/or aromatic residues at this position. These results suggest aa293 is very important for signal perception or signal transduction. According to secondary structure prediction of the VirA linker domain, aa293 is located in a loop just before a critical helix coiled coil that regulates signal transmission (Wang *et al.* 2002). In the next chapter, I extend the study of aa293 and the loop where aa293 resides.

Table 3.3 Inhibition of VirA kinase activity by overexpression of the receiver domain aa712-829 (Chang *et al.* 1996).

VirA construct	Receiver module	β- Galactosidase activity (Miller unit)
		No AS
VirAΔ712-829	none	565
VirAΔ712-829	712-829	20
VirAΔ712-829	712-829(D766N)	7

Figure 3.12 Schematic representation of the (Δ408ThkA)$_2$/2TrrA complex (Yamada *et al.* 2006). (a) Domain-assigned map: sky-blue, PAS-sensor domain; yellow, DHp (dimerization/histidine-containing phosphotransfer) domain; green, CA (ATP-binding/catalytic) domain; red, TrrA (RR). (b) With ribbon representation where RR-A (red), RR-B (blue), PAS-A (magenta) and PAS-B (cyan).



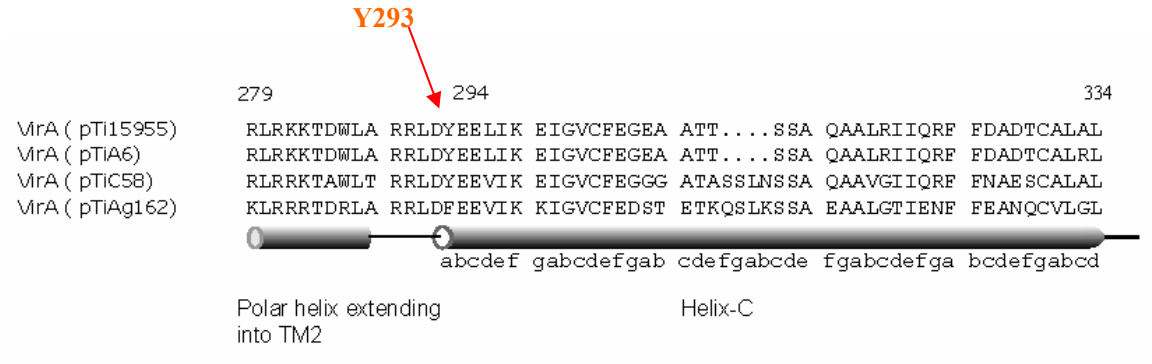
CHAPTER 4

Genetic Evidence for Phenol Specificity Switch Caused by the Region aa288-aa293 of VirA Protein in *Agrobacterium tumefaciens*

Introduction

In Chapter 3, I demonstrated that compensating suppressor mutants for receiver nulls could be dramatic and unexpected. For example, LKR(285-829)(Y293F) dramatically increases the sensitivity and LKR(285-829)(Y293F/V765D) significantly enhances both the sensitivity and maximal response to AS. Therefore, it appears that aa293 is critical for signal regulation in the VirA/VirG two-component system. From the secondary structure predictions, aa293 is located just before a highly conserved amphipathic helix on the C-terminal side of TM2 and at the start of Helix C (Fig 4.1). It has been reported that when membrane-spanning and periplasmic domains of VirA were replaced with the GCN4 leucine zipper at aa294, the GCN4 fusions conformationally bias the interface of the Helix C coiled coil in the VirA dimer. The activity of these constructs LZ(0/3/4)-LKR(294-829) has been argued to be dependent on the interface of the Helix C coiled coil (Wang *et al* 2002). Based on these results, a ratchet model has been proposed the VirA activation is achieved by signal-induced switching of the interfaces of the homodimer (Wang *et al*. 2002). Furthermore, genetic deletion and mutation data have implicated the region in or near TM2 in signal inputs (Toyoda-Yamamoto *et al*. 2000; Melchers *et al*. 1989; Doty *et al*. 1996). It is therefore possible

Figure 4.1 Sequence alignments of four VirA sequences, shown are TM2 helix, the helix C with the Coiled coil designated, and the loop region before the helices. The numbering corresponds to the VirA (pTiA6) sequence.



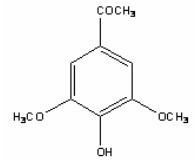
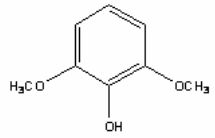
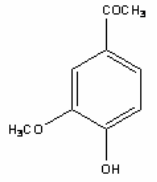
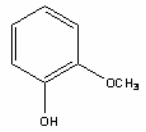
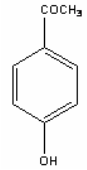
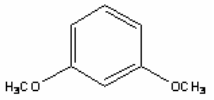
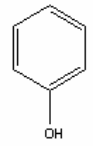
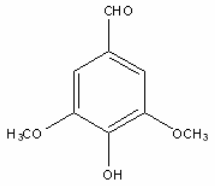
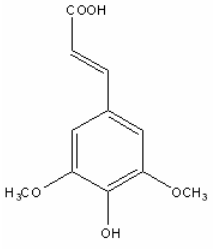
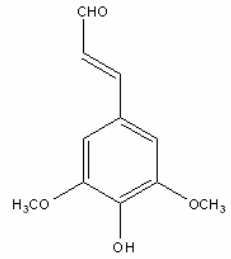
that the residues in the short region before aa294, including aa293, are involved in phenol perception for changing the interface of the helix coiled coil.

A. tumefaciens is a multi-host pathogen, and a structurally diverse group of plant-derived phenolic compounds are detected by VirA to induce the virulence response. The structural features of phenolic compounds have been analyzed by testing the *vir*-inducing ability of over 100 compounds. A phenol with one or two ortho methoxy substituents is required, while a less strict requirement is for an acyl group at the para position (Melchers *et al*. 1989). It remains intriguing how a single receptor site on VirA can sense these various phenolic compounds. In this chapter, I focused on studying aa293 and the short region aa285-293 at the N-terminus of linker domain to better understand this broad structural specificity. I discovered that changes across aa288-293 are able to switch the specificity and remove the requirement for the para acetyl group. Remarkably, single mutations such as Y293T in this region greatly reduce the range of known *vir* inducers, suggesting this region may be important for directing *Agrobacterium tumefaciens'* range. These alleles will now enable direct tests of the importance of responding to many different signals in multi-host pathogenesis.

Material and methods

Bacterial Strains, Plasmids and Reagents. Acetosyringone (AS), 2,6-Dimethoxyphenol (2, 6-DMP), Acetovanillone (AV), Guaiacol (GA), 4-Hydroxyacetophenone (HAP), 2,6-Dimethoxybenzene (1, 3-DMB), Phenol, Syringaldehyde (SAE), Sinapinic acid (SPA), Sinapinaldehyde (SPAE) listed in Table 4.1 used for *vir* induction assays were purchased from Sigma-Aldrich Corp. 3,5-dimethoxyaniline, squaric acid, 3-methoxypyridine and

Table 4.1 The structures of phenols used Chapter 4.

Acetosyringone (AS) 	2,6-Dimethoxyphenol (2, 6-DMP) 
Acetovanillone (AV) 	Guaiacol (GA) 
4-Hydroxyacetophenone (HAP) 	2,6-Dimethoxybenzene (1, 3-DMB) 
Phenol 	Syringaldehyde (SAE) 
Sinapinic acid (SPA) 	Sinapinaldehyde (SPAE) 

3, 5-dimethoxypyridine used for library screen listed in Table 4.5 were purchased from Sigma-Aldrich Corp. The enzymes for DNA manipulation were purchased from either New England Biolab or Promega unless specified. The bacterial strains and plasmids used in this study are listed in Table 4.2. *E. coli* strain XL1-Blue was used for routine cloning.

Plasmid constructions. Each truncated and site specifically altered VirA gene was introduced into pJZ6, a vector that has unique BamH I and Acc65 I restriction cutting sites and can replicate both in *E. coli* and *A. tumefaciens*. To create the VirA allele LKR(280-829), PCR was used to amplify the product LKR(280-829) from the template pYW48 with primers LKR280 and LKRA. The fragment was digested and cloned into the BamH I and Acc65 I sites of pJZ6 to give plasmid pQF375. Following the same method, PCR products were amplified from pYW48 with one variable primer corresponding to different truncated VirA (LKR286, LKR287, LKR288, LKR289, LKR290, LKR291, LKR292, LKR293, LKRF and LKR295) and LKRA1.The resulting digested fragments LKR(286-829), LKR(287-829), LKR(288-829), LKR(289-829), LKR(290-829), LKR(291-829), LKR(292-829), LKR(293-829), LKR(294-829), LKR(295-829) were cloned into pJZ6 to give plasmids pQF410, pQF411, pQF412, pQF413, pQF414, pQF415, pQF416, pFQ139 , pQF417 and pQF418 respectively. To create VirA alleles LK(285-711), LK(287-711), LK(288-711), LK(293-711), LK(294-711) and LK(285-711)(Y293T), PCR was used to amplify the products from pYW48 or pQF282 with primers LKRF, LKR287F, LKR288F, LKR293F, LKR294F and antisense primer LK6HISR. To construct LKR(285-829) with different mutations, recombinant PCR was used to amplify LKR(285-829) with primers LKRF, LKRA and primers

Table 4.2 Bacterial strains and plasmids used in Chapter 4.

Strains/plasmids	Relevant characteristics	Reference
E. coli.		
XL1-Blue	*recA1 endA1 gyrA96 thi⁻1 hsdR17 supE44 relA1 lac[F' proAB lacI^{q}Z M15 Tn 10* (Tc^r)*]A*	Stratagene
SU101	P*sulA*-*lacZ* fusion with operator site (op408)	(Daines *et al*. 2000)
A .tumefaciens		
A136	Strain C58 cured of pTi plasmid	(Watson *et al*. 1975)
A348-3	C58 background with pTiA6NC, *virA*::*kan^r*	(Lee *et al*. 1992)
Plasmids		
pYW15b	Broad-host-range expression vector, IncW, Ap^r	(Wang *et al*. 2000)
pYW61b	P_{N25}:LZ(0)-LKR in pYW15b	(Wang *et al*. 2002)
pRG109	IncP plasmid carrying P*virB*-*lacZ* fusion and P_{N25}-*virG*, *Spec^r*	(Gao *et al*. 2005)
pSW209Ω	IncP plasmid carrying P*virB*-*lacZ* fusion, *Spec^r*	(Campbell *et al*. 2000)
pJZ6	IncW/ColE expression vector with P_{N25}, *Carb^r*	(Zhang *et al*. 2000)
pYW34	pYW15b carrying *virA* LKR (285-829), *Carb^r*	(Wang *et al*. 1999)

Strains/plasmids	Relevant characteristics	Reference
pYW48	pYW15b carrying *virA* (1-829), *Carb*^{r}	(Wang *et al*. 1999)
pQF375	pJZ6 carrying VirA truncation LKR(280-829), *Carb*^{r}	This study
pQF410	pJZ6 carrying VirA truncation LKR(286-829), *Carb*^{r}	This study
pQF411	pJZ6 carrying VirA truncation LKR(287-829), *Carb*^{r}	This study
pQF412	pJZ6 carrying VirA truncation LKR(288-829), *Carb*^{r}	This study
pQF413	pJZ6 carrying VirA truncation LKR(289-829), *Carb*^{r}	This study
pQF414	pJZ6 carrying VirA truncation LKR(290-829), *Carb*^{r}	This study
pQF415	pJZ6 carrying VirA truncation LKR(291-829), *Carb*^{r}	This study
pQF416	pJZ6 carrying VirA truncation LKR(292-829), *Carb*^{r}	This study
pFQ139	pJZ6 carrying VirA truncation LKR(293-829), *Carb*^{r}	This study

Strains/plasmids	Relevant characteristics	Reference
pQF417	pJZ6 carrying VirA truncation LKR(294-829), *Carb*^{r}	This study
pQF418	pJZ6 carrying VirA truncation LKR(295-829), *Carb*^{r}	This study
pQF289	pJZ6 carrying VirA allele LKR(285-829)(R290Y), *Carb*^{r}	This study
pQF282	pJZ6 carrying VirA allele LKR(285-829)(Y293T), *Carb*^{r}	This study
pQF419	pJZ6 carrying VirA truncation LK(287-711)-6His, *Carb*^{r}	This study
pQF420	pJZ6 carrying VirA truncation LK(288-711) - 6His, *Carb*^{r}	This study
pQF421	pJZ6 carrying VirA truncation LK(293-711) - 6His, *Carb*^{r}	This study
pQF422	pJZ6 carrying VirA truncation LK(294-711) - 6His, *Carb*^{r}	This study
pQF314	pJZ6 carrying VirA LK(294-711)(Y293T) - 6His, *Carb*^{r}	This study
pQF267	pJZ6 carrying VirA allele LKR(285-829)(Y293P), *Carb*^{r}	This study

Strains/plasmids	Relevant characteristics	Reference
pQF295	pJZ6 carrying VirA allele LKR(285-829)(Y293S), *Carb*^{r}	This study
pQF281	pJZ6 carrying VirA allele LKR(285-829)(D292V), *Carb*^{r}	This study
pQF285	pJZ6 carrying VirA allele LKR(285-829)(R290G), *Carb*^{r}	This study
pSR658	Wild-type LexADBD fusion vector, *Tet*^{r}	(Elderkin *et al*. 2005)
pQF425	pSR658 carrying LZ-L(294-449), *Tet*^{r}	This study
pQF426	pSR658 carrying L(285-449), *Tet*^{r}	This study
pQF427	pSR658 carrying L(294-449), *Tet*^{r}	This study
pQF428	pSR658 carrying LK(285-449), *Tet*^{r}	This study
pQF429	pSR658 carrying LK(294-449), *Tet*^{r}	This study
pQF229	pRG109 carrying VirA truncation LK(287-829)-6His, *Spec*^{r}	This study

Table 4.3 Primers used in Chapter 4.

Primer Name	Sequence	Characteristics
LKRF	CG *GGATCC* GAA GAG CTA ATC AAA GAG	BamH I & Sense
LKRA	GC *GGTACC* GCA ACT CTA CGT CTT GAT	Kpn I & antiSense
LKR280	CG *GGATCC* CTA CGC AAA AAA ACC GAT	BamH I & Sense
LKR286	CG *GGATCC* TGG TTA GCG CGG CGT	BamH I & Sense
LKR287	CG *GGATCC* TTA GCG CGG CGT TTA	BamH I & Sense
LKR288	CG *GGATCC* GCG CGG CGT TTA GAT	BamH I & Sense
LKR289	CG *GGATCC* CGG CGT TTA GAT TAC	BamH I & Sense
LKR290	CG *GGATCC* CGT TTA GAT TAC GAA	BamH I & Sense
LKR291	CG *GGATCC* TTA GAT TAC GAA GAG	BamH I & Sense

Primer Name	Sequence	Characteristics
LKR292	CG *GGATCC* GAT TAC GAA GAG CTA	BamH I & Sense
LKR293	CG *GGATCC* TAC GAA GAG CTA ATC	BamH I & Sense
LKR295	CG *GGATCC* GAG CTA ATC AAA GAG	BamH I & Sense
LK6HISR	GG *GGTACC* CTA GTG GTG GTG GTG GTG GTG ACG CGG TGC CTT ATT GCG	Kpn I & antiSense
Y293PF	CG *GGATCC* GAT TGG TTA GCG CGG CGT TTA GAT CCC GAA	Y293P & BamH I
Y293SF	CG *GGATCC* GAT TGG TTA GCG CGG CGT TTA GAT TCC GAA	Y293S & BamH I
Y293TF	CG *GGATCC* GAT TGG TTA GCG CGG CGT TTA GAT ACC GAA	Y293T & BamH I
D292VF	CG *GGATCC* GAT TGG TTA GCG CGG CGT TTA GT T TAC	D292V & BamH I
R290GF	CG *GGATCC* GAT TGG TTA GCG CGG *GGT* TTA	R290G & BamH I
L449RKPNI	GC *GGTACC* CTA GCA TTC GGT CTG CTT ACG	Kpn I & antiSense

Primer Name	Sequence	Characteristics
LZF	GC *GAGCTC* GGA GGT TGC GGA GGT AAG	Sac I & Sense
LKRSacI	CG *GAGCTC* GAT TGG TTA GCG CGG CGT	Sac I & Sense
LKKPNI	GG *GGTACC* CTA ACG CGG TGC CTT ATT GCG	Kpn I & antiSense
LKRS	GC *GAGCTC* GAA GAG CTA ATC AAA GAG	Sac I & Sense

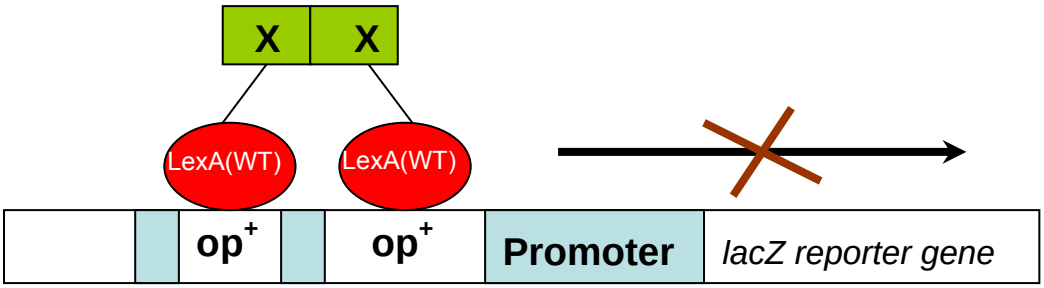
carrying the substitute in the positions listed in Table 4.3. These fragments were digested and cloned into BamH I and Acc65 I sites of pJZ6. The resulting plasmids are pQF267, pQF281, pQF285, pQF282 and pQF295 carrying mutations Y293P, D292V, R290G, Y293T and Y293S respectively. To construct plasmids pQF425, pQF426, pQF427, pQF428 and pQF429, fragment LZ-L(294-449) was amplified using PCR with pYW61b as template and primers LZF and L449RKPNI. Meanwhile fragments L(285-449) and L(294-449) were amplified using PCR with pYW48 as template and sense primers LKRSac I, LKRS and antisense primer L449RKPNI respectively. Fragments LK(285-711) and LK(294-711) were amplified using PCR with pYW48 as template and sense primers LKRSacI, LKRS and antisense primer LKPNI. These PCR products were digested with Sac I and Kpn I and inserted into pSR658. All PCR product sequences above were confirmed by DNA sequencing. The plasmids in pJZ6 or pYW15b shuttle

vectors were introduced into *A. tumefaciens* A136/pRG109 carrying P_{virB}*:lacZ* and P_{N25}*:virG* or A348-3/pSW209Ω carrying P_{vir}*:virG* by eletroporation. The plasmids in the vector pSR658 were transformed into *E. coli* strain SU101.

Library screens for specificity switch alleles. Random mutagenesis was carried out in the region aa285-aa294 and then truncated LKR alleles were ligated into pJZ6 using BamH I and Acc65 I restriction enzymes. The ligation mixture was introduced into A136/pRG109 by eletroporation. The transformants were poured on the induction medium plates with X-gal and 100μM AS, compared with the wild type LKR(285-829) in A136/pRG109 on the same IM plates, and the white colonies were pooled and replicated on the IM plates with X-gal and 100μM 2, 6-DMP. The colonies with bright blue color were picked and amplified by colony PCR. Mutations were identified by DNA sequencing and confirmed by site-directed mutagenesis.

Protein homodimerization assay. The LexA-based bacterial protein interaction system described in Fig 4.2 used in this work has been described preciously (Dmitrova *et al*. 1998; Daines *et al*. 2000). *E. coli* strains SU101 carrying pQF425, pQF426, pQF427, pQF428, pQF429 or pSR658 were grown overnight at 37^{o}C with 1mM IPTG and the appropriate antibiotics. These LB cultures were diluted 60-fold into LB medium containing 1mM IPTG and the antibiotics, and β-galactosidase activity was determined at an OD600 between 0.4-0.6 (Miller *et al*. 1972). Protein-Protein association was determined by the LexA-based bacterial protein interaction system (Daines *et al*. 2000) and quantified by the repression of the *sulA::lacZ* reporter. The β-galactosidase activity

Fig 4.2 The LexA repressor-based bacterial two-hybrid system. For homodimer interaction assay, X represents a protein fused to wild type LexA DBD, the interaction between X and X produces a homodimer capable of binding to the operator sites and repressing *lacZ* reporter gene expression.



produced by the strain carrying the pSR658 vector was set as maximal activity and repression was defined as 1-(β-galactosidase activity of sample)/(maximal activity).

Immunoblot analysis. The method was described in Chapter 3 to detect the protein expression of LKR alleles in *A. tumefaciens*. For checking total protein expression in *E. coli* strain SU101, the cells were grown for 4hr after OD_{600} reaches 0.4-0.6, harvested and the pellet was dissolved in 2xSDS loading buffer and boiled for 10mins. The supernatant was collected after a 20 min spin at 14000 rpm, diluted to 1xSDS solution for loading into SDS-PAGE gels and transferred to the nitrocellulose membrane. The membrane was probed with anti-lexA at 1:2500 dilution (Invitrogen), followed with anti-rabbit antibody conjugated to alkaline phosphatase (Amersham) at 1:1000 dilution, and visualized by 1-Step NBT/BCIP (Pierce) developing reagent.

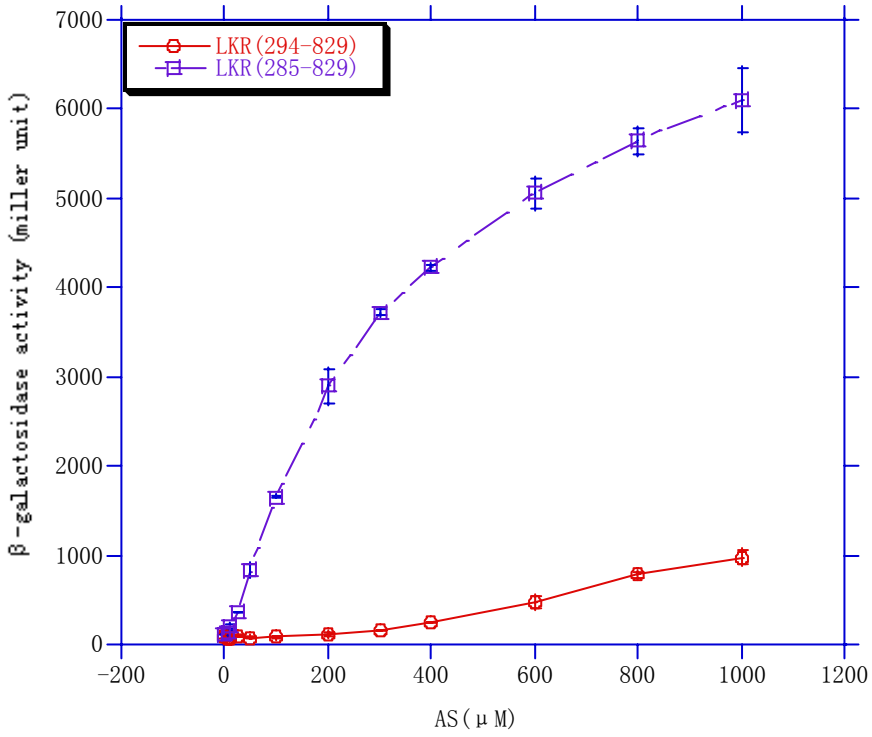
Results

LKR(285-829) and LKR(294-829) respond very differently to AS induction

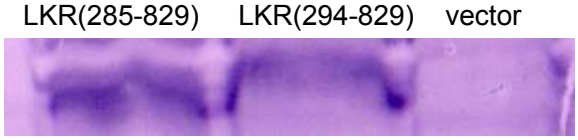
To test whether the short region aa285-293 affects phenols perception or not, this region was deleted and VirA alleles LKR(285-829) and LKR(294-829) were tested for AS response. Comparing the AS induced activity of LKR(285-829) with that of LKR(294-829), LKR(294-829) responds to AS much more weakly than LKR(285-829). As shown in Fig 4.3A, with overexpressed VirG, LKR(294-829) is only activated at the concentration of AS (600μM) while LKR(285-829) is activated at much lower concentration of AS (around 20μM). The protein expression level is almost equivalent (Fig 4.3 B) indicating it is not the factor to lead to the dramatic activation difference.

Fig 4.3 LKR(294-829) responds to AS very differently with LKR(285-829). (A) Derivatives of *A. tumefaciens* A136/pRG109 carrying the indicated plasmids pYW34, and pFQ144 were cultured in induction medium with different concentrations of AS and assayed for the *vir* gene expression as described in the method. (B) Western blot analysis using the VirA antibody. lane1, LKR(285-829); lane2, LKR(294-829); lane3, vector control lacking VirA construct.

(A)



(B)

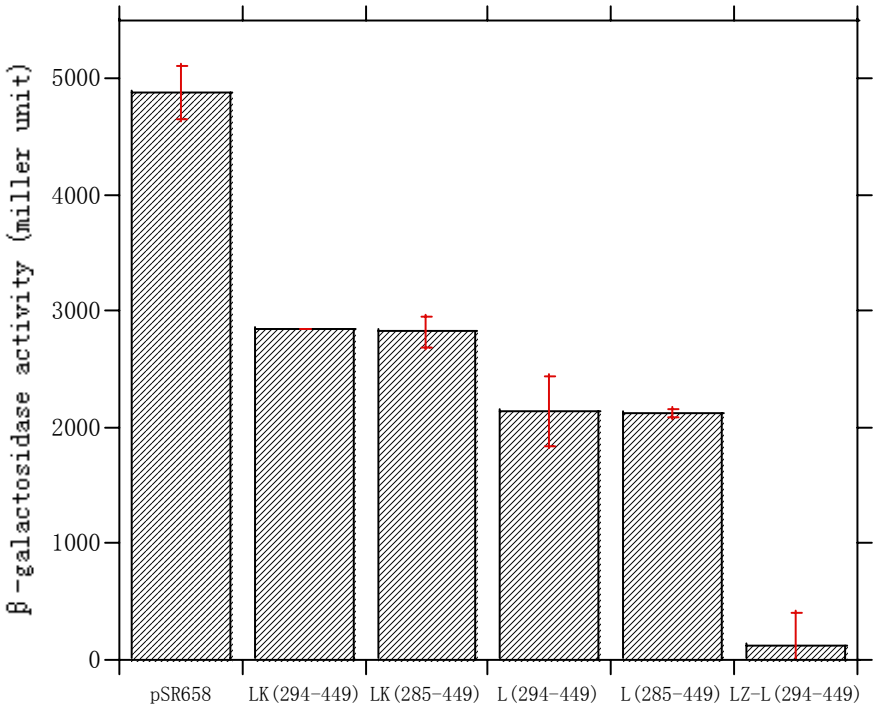


Does aa285-aa293 affect VirA dimer formation?

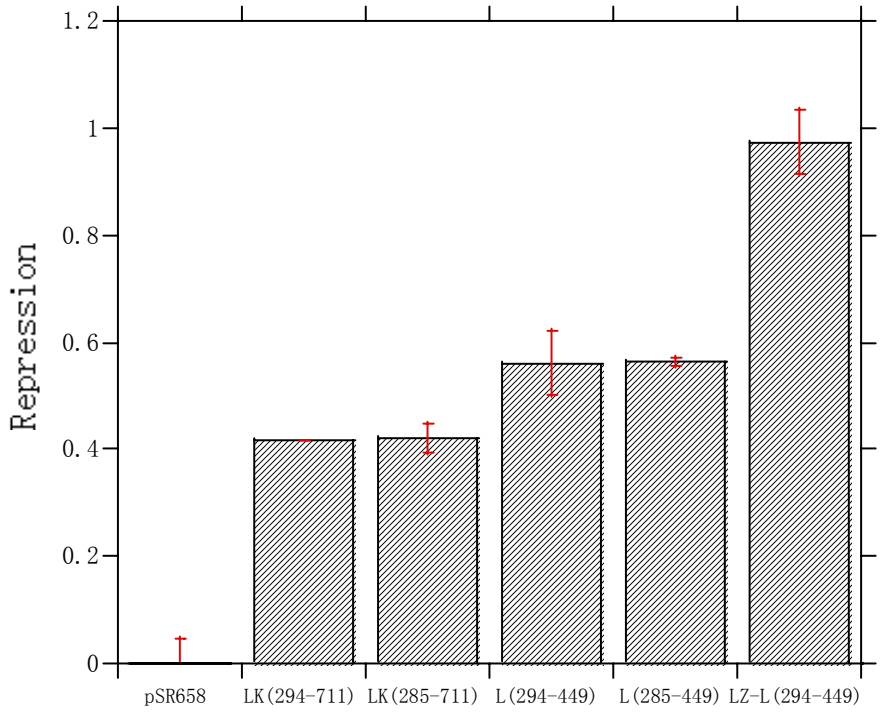
Signal-mediated activation of HSKs such as VirA requires dimer formation. Indeed, the evidence that LZ stabilizes LZ(TM2)-LKR (G665D) significantly increases both basal *vir* gene expression (without AS) and a maximal *vir* gene expression (with 100 μM AS) suggests dimerization of VirA is important for its in vivo activity. Secondary structure analysis and previous GCN4 fusions (Wang *et al* 2002) suggested that even though LKR(294-829) is inactive, GCN4 is able to favor an activity with proper fusions. Therefore, aa285-293 must have some activating function. To test if this activating function involves the induction of dimerization, the bacterial two-hybrid system based on the LexA repressor (Clarke *et al*. 2005) was used to analyze protein homodimer formation. The LexA protein consists of two domains, an N-terminal DBD and a C-terminal domain responsible for dimerization. Both of these domains are necessary for LexA to function as a repressor (Clarke *et al*. 2005). In the two-hybrid experiment, genes encoding the linker domain or linker with kinase domain starting from aa285 and aa294 were used to replace the C- terminal domain of wild type LexA carried in pSR658. Hybrid proteins capable of forming multimers result in the formation of a functional LexA DBD dimer, which inhibits expression of a *sulA::lacZ* gene fusion in the *E. coli* SU101 reporter strain (Kiratisin *et al*. 2002). As shown in Fig 4.4A, pSR658 without the dimerization domain shows very high activity while with starting from either aa285 or aa294 also has the same ability of repression, 50%. Western blots confirm that both constructs were expressed at the same level (Fig 4.4C).The addition of the leucine zipper, LZ-L(294-449), indeed has a stronger potential to dimerize, consistent with the evidence that LZ stabilizes

Fig 4.4 The region aa285-293 does not affect VirA dimerization. *E. coli* strain SU101 (*sulA:: lacZ*) carrying the vector pSR658 or the constructs indicated as below. lane1: pSR658; lane2, LK(294-711) in pSR658; lane3, LK(285-711) in pSR658; lane4, L(294-426) in pSR658; lane5, L(285-426) in pSR658 and lane6, LZ-L(294-426) in pSR658. (A) The *sulA::lacZ* expression in each strain. (B) Calculated as repression of *sulA::lacZ* expression. (C) Western blot analysis with the anti-lexA antibody.

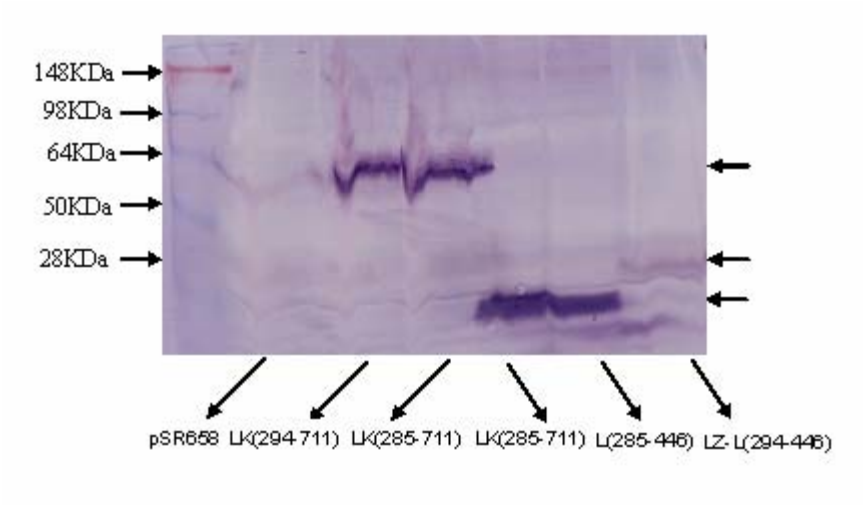
(A)


β-galactosidase activity (miller unit)
5000
4000
3000
2000
1000
0
pSR658
LK(294-449)
LK(285-449)
L(294-449)
L(285-449)
LZ-L(294-449)

(B)


Repression
1.2
1
0.8
0.6
0.4
0.2
0
pSR658
LK(294-711)
LK(285-711)
L(294-449)
L(285-449)
LZ-L(294-449)

(C)


148KDa
98KDa
64KDa
50KDa
28KDa
pSR658
LK(294-711)
LK(285-711)
LK(285-711)
L(285-446)
LZ-L(294-446)

VirA dimerization (Fig 4.4B).The results of the bacterial two-hybrid experiments therefore indicate that the linker starting from aa294 and aa285 has equal potential to dimerize, but its in vivo activity is weaker than LKR(285-829). Therefore, aa285-293 must be regulating something other than simple dimerization.

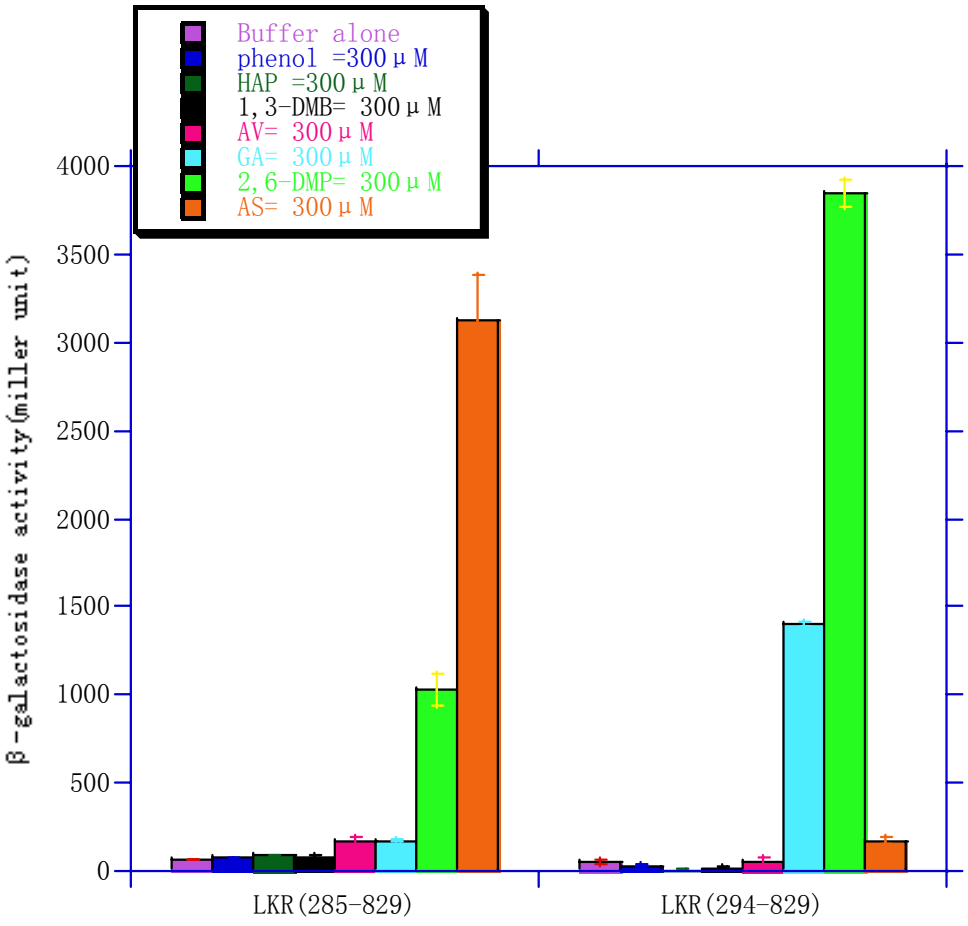
Does aa285-aa293 regulate phenol specificity?

Based on the evidence above, protein expression and dimerization are not primary factors reducing LKR(294-829) activation by AS. There is only one explanation left that is the region aa285-293 involves in phenols recognition. To test whether the region aa285-293 is critical for phenol recognition, a series of phenol analogs were investigated. According to the structure-activity analysis of AS, the phenol with two ortho-methoxyl groups and a para acetyl group all contribute to the VirA activation. VirA truncation LKR(294-829) with P_{N25} driven virG displayed a significant shift in structural specificity related to wild type LKR(285-829). As shown in Fig 4.5, LKR(294-829) only responds to GA and 2, 6-DMP, compounds lacking the para acetyl substitute. Apparently, aa285-aa293 is responsible for a complete switch in specificity when the generic inducer is no longer active.

Are specific residues across the region aa285-293 responsible for the phenol specificity switch?

Two approaches were taken to identify critical residues across aa285-aa294 responsible for specificity. Firstly, the gene sequence encoding aa285-aa294 was used as a template for amplification with random mutagenesis by PCR. The resulting library was

Fig 4.5 The region aa285-293 regulates specificity. Derivatives of *A. tumefaciens* A136/pRG109 carrying indicated plasmids pYW34 and pFQ144 were cultured in induction medium with either buffer alone or 300μM inducers (phenol, HAP, 1, 3-DMB, AV, GA, 2, 6-DMP and AS) and assayed for the *vir* gene expression as described.



inserted into a shuttle vector pJZ6 and transformed into *Agrobacterium* strain A136/pRG109. The transformants were screened on induction medium plates with X-gal and 100 μM AS for white colonies (no response to AS), and the white colonies were pooled and poured on the induction medium plates with X-gal and 100 μM 2, 6-DMP to screen for blue colonies (good response to 2, 6-DMP). Selected colonies that switch phenol specificity were cultured and the alleles were sequenced. As shown in Fig 4.6 and Fig 4.7, most of the mutants carried changes were in the region aa290-aa294 and LKR(285-829)(Y293T) displayed the most similar phenotype as LKR(294-829). Secondary structure prediction identified an amphipathic helix (aa280-aa287) and a flexible loop (aa288-aa293) just after TM2 and before the Helix-C coiled coil, providing the basis for the original LZ fusions (Wang *et al*. 2002). Based on the secondary structure analysis, in the second approach to resolve the specificity, a series of LKR truncations were constructed and tested for *vir* gene induction with AS and 2, 6-DMP (Fig 4.8). Although the maximal *vir* gene induction changes with stepwise truncation, AS proved to be a more active inducer between LKR (280-829) and LKR (287-829) while between LKR (288-829) and LKR (294-829), 2, 6-DMP is the more active inducer, especially the truncated VirA LKR (294-829) only responds to 2, 6-DMP. LKR (295-829) is found to have much lower maximal *vir* induction than LKR (294-829) with 2, 6- DMP, suggesting that interrupted helix-coil severely affects the signal transduction. Western blot analysis using anti-VirA polyclonal antibody shows that all VirA truncation proteins are stably expressed (data not shown). Both truncation and library screen results indicate that residues across the region aa288-294 are responsible for specificity switch.

Fig 4.6 Specific residues across aa285-293 are responsible for specificity switch. Derivatives of *A. tumefaciens* A136/pRG109 carrying indicated plasmids pQF285, pQF281, pQF282, pQF267, pQF295, pYW34 and pFQ144 were cultured in induction medium with either buffer alone or 300 µM inducers (2, 6-DMP and AS) and assayed for the *vir* gene expression as described.

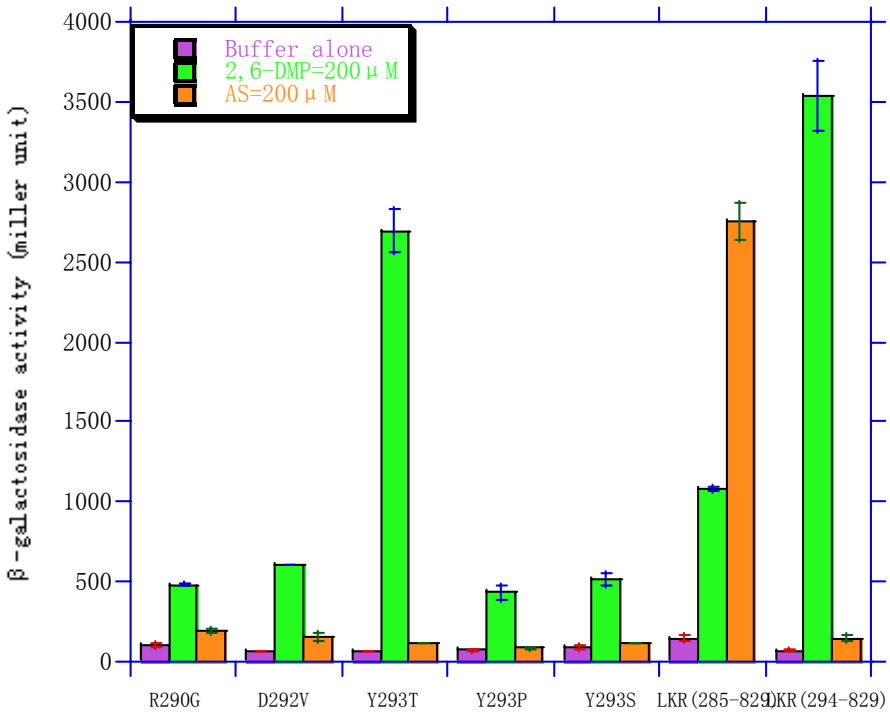


Fig 4.7 LKR(285-829)(Y293T) regulates specificity. Derivatives of *A. tumefaciens* A136/pRG109 carrying indicated plasmids pYW34 and pFQ282 were cultured in induction medium with either buffer alone or 300 µM inducers (phenol, HAP,1, 3-DMB, AV, GA, 2, 6-DMP and AS) and assayed for the *vir* gene expression as described.

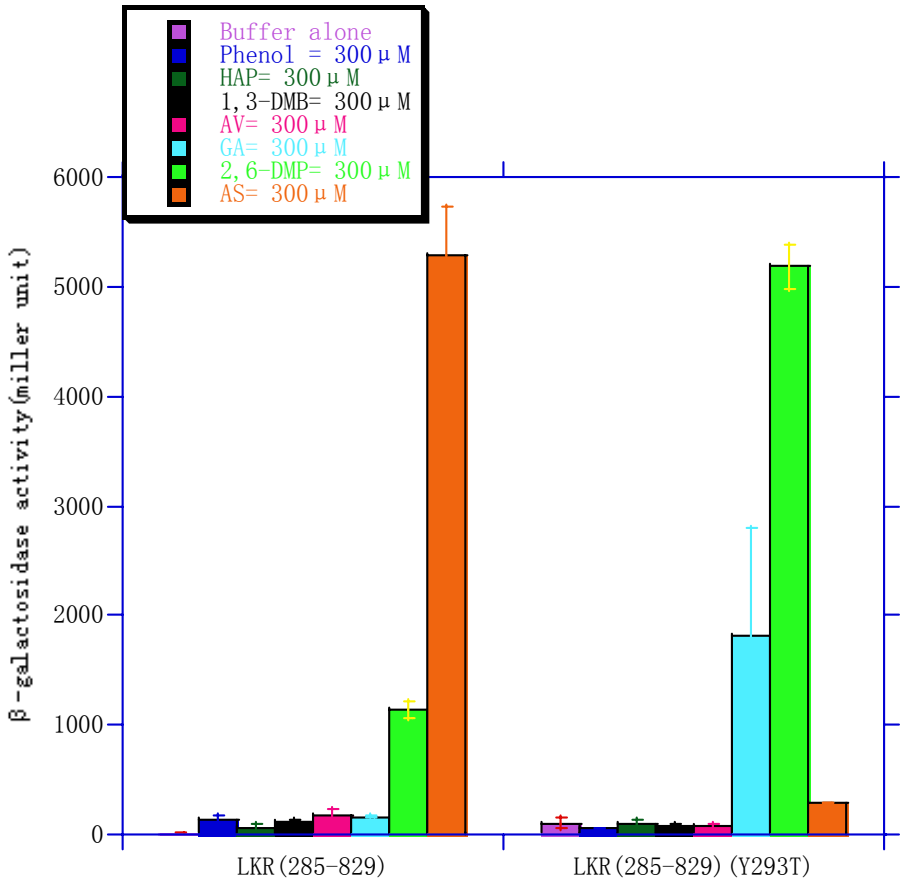
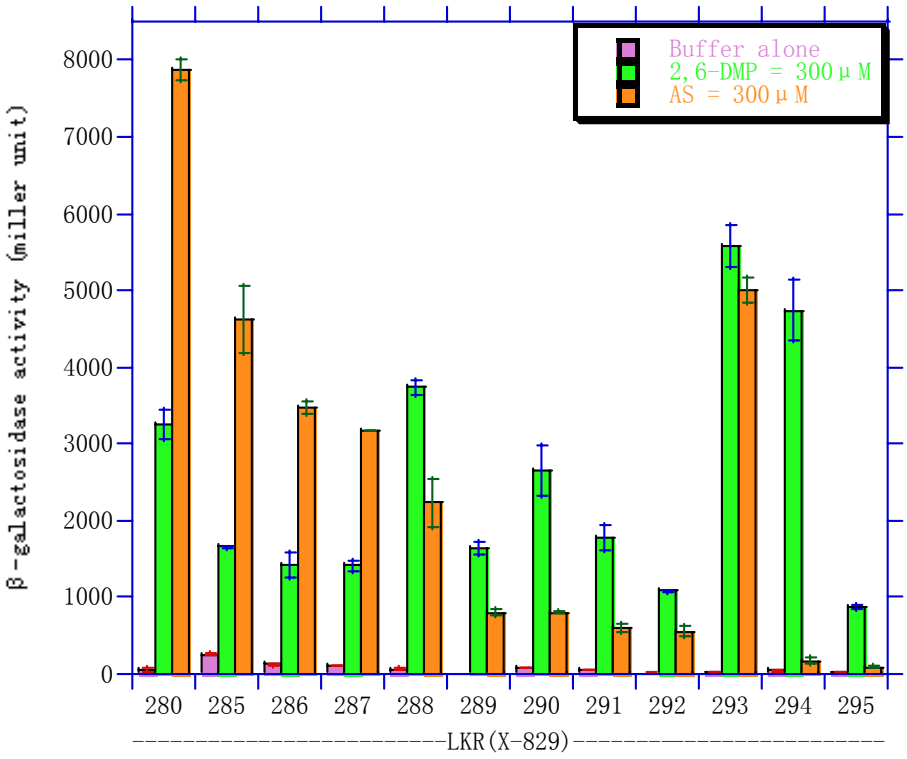


Fig 4.8 The region aa288-293 regulates specificity switch. Derivatives of *A. tumefaciens* A136/pRG109 carrying indicated plasmids pQF375, pYW34, pQF410, pQF411, pQF412, pQF413, pQF414, pQF415, pQF416, pQF417, pFQ144 and pQF418, were cultured in induction medium with either buffer alone or 300 μM inducers (2, 6-DMP and AS) and assayed for the *vir* gene expression as described.



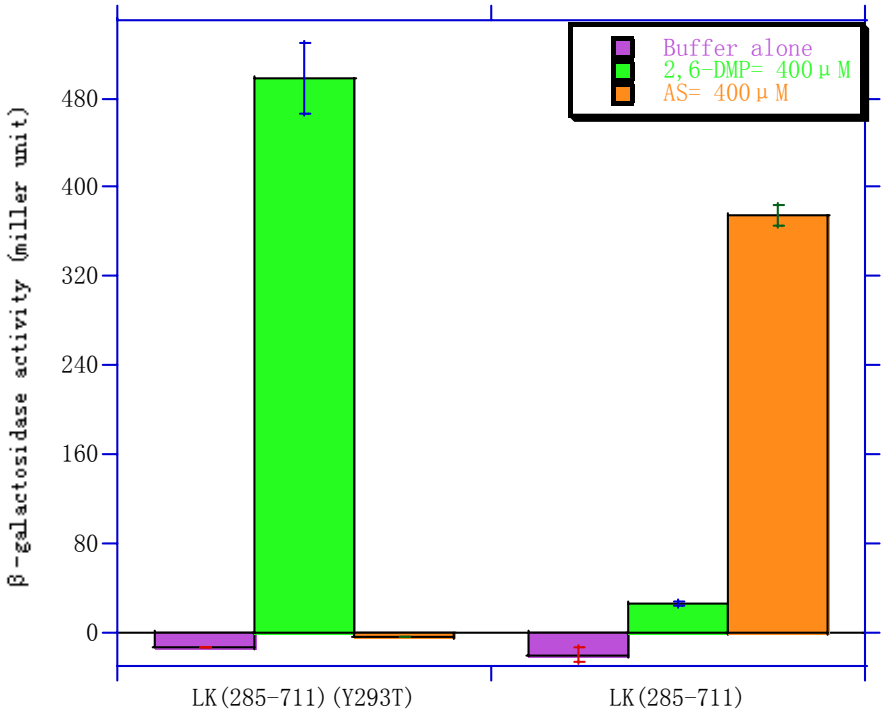
The presence of receiver domain of VirA is not involved in the specificity

As discussed in Chapter 3, the linker and the receiver function cooperatively and the association appears to be regulated by the phenols. Moreover, VirA with mutations (S778P or I734N) in the receiver domain respond to weak inducer HAP suggesting that receiver domain likely influences the phenol recognition (Chang *et al*. 1996). To test whether VirA receiver domain is responsible for the phenol specificity change, VirA alleles LK(287-711), LK(288-711), LK(293-711), LK(294-711) and LK(285-711)(Y293T) were tested for AS and 2, 6-DMP induction. They had the same response to AS and 2, 6-DMP with and without receiver domain shown in Fig 4.9 indicating that receiver domain is not involved the phenol specificity change.

Maximal activity change is the determinant for phenol specificity

AS and 2, 6-DMP dose response assays with different LKR truncations and overexpressed VirG show that in each truncation, the concentration (ED_{50}) of inducers (AS and 2, 6-DMP) to reach the half maximal activity is similar except that LKR(294-829) is almost dead in different concentrations of AS, however, the maximal activity changes a lot with different truncations shown in Table 4.4. For LKR(287-829), the activation by AS (5800 miller unit) is higher than that by 2, 6-DMP (3200 miller unit) which is similar as LKR(285-829) while for LKR(288-829), the activation by AS (5800 miller unit) is similar as that by 2, 6-DMP (5800 miller unit); especially for LKR(294-829), the activation by AS (1000 miller unit) is one seventh of that by 2, 6-DMP (7000 miller unit). This data suggests that maximal activity change to AS and 2, 6-DMP induction for different truncations leads to the phenol specificity change.

Fig 4.9 The VirA receiver domain is not responsible for specificity. Derivatives of *A. tumefaciens* A348-3/pSW209Ω carrying the plasmid pQF314, pFQ282, pQF411, pQF412, pQF417 and pFQ144 were cultured in induction medium with either buffer alone or 400 μM inducers (2, 6-DMP and AS) and assayed for the *vir* gene expression as described.


Buffer alone
2, 6-DMP= 400 μ M
AS= 400 μ M
β-galactosidase activity (miller unit)
480
400
320
240
160
80
0
LK(285-711) (Y293T)
LK(285-711)

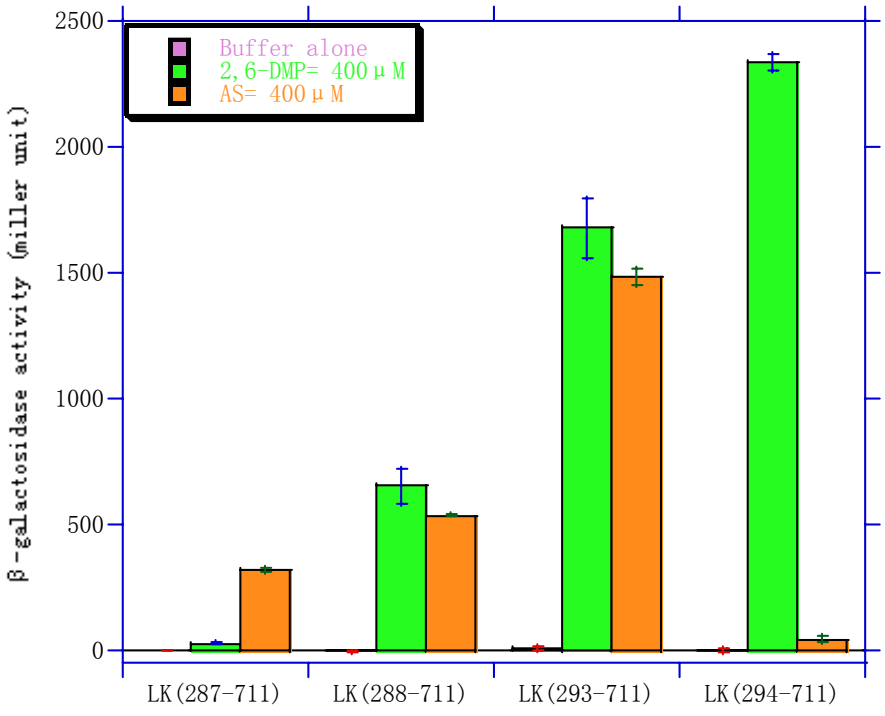

Buffer alone
2, 6-DMP= 400 μ M
AS= 400 μ M
β-galactosidase activity (miller unit)
2500
2000
1500
1000
500
0
LK(287-711)
LK(288-711)
LK(293-711)
LK(294-711)

Table 4.4 ED_{50} and maximal activity of VirA truncation alleles to AS and 2, 6-DMP induction.

Strains[a]	$ED_{50}(\mu M)$[b]		Maximal activity(miller unit)[c]	
	AS	2, 6-DMP	AS	2, 6-DMP
LKR(287-829)	300	300	5800	3200
LKR(288-829)	350	250	5800	5800
LKR(293-829)	300	250	8000	7500
LKR(294-829)	>600 N/A	300	1000	7000

a. Strain A136/pRG109 containing LKR(287-829), LKR(288-829), LKR(293-829) and LKR(294-829) (from top to bottom) were assayed.

b. ED_{50} was determined by fitting the AS and 2, 6-DMP induction curve as described in Methods.

c. Maximal activity was determined with 1mM AS and 2, 6-DMP induction.

Residues across aa285-293 control specificity for para position

Library screens were also utilized to select the residues in the region aa285-aa293 for phenol specificity switch related with other groups in the phenolic compounds. As described in the method, the segment of aa285-293 as a temple was amplified by random mutagenesis PCR and inserted into a shuttle vector pJZ6 and transformed into *Agrobacterium* strain. The transformants were screened on induction medium plates with X-gal and 100 μM AS for white colonies (no response to AS), and white colonies were pooled and poured on the induction medium plates with X-gal and 100 μM phenolic compounds listed in Table 4.5 individually for blue colonies (good response to phenolic compounds). However, no blue colonies have yet been found in these libraries. Although it is likely that the libraries are not big enough to select the mutations, it implies that the region aa285-293 is only critical for specificity switch related with the para acetyl group in the phenolic compounds.

Y293T dramatically narrows specificity

Agrobacterium tumefaciens appears to detect phenols signals such as ferulic acid, syringaldehyde and coniferylalcohol as characteristic of all dicotyledonous plants (Melchers *et al*. 1989). Since *Agrobacterium tumefaciens* initiates pathogenesis in a wild host range although the phenol recognition strategy of VirA remains unknown, the strain with LKR(285-829)(Y293T) was chosen and *vir* induction was assayed using inducers with different groups at the para position shown in Fig 4.10. For LKR(285-829), at the concentration of 200μM inducers, it responds to acetosyringone (AS), sinapinic acid (SPA), syringaldehyde (SAE) and sinapinaldehyde (SPAE) as well as wild type VirA

Table 4.5 Compounds for library screen

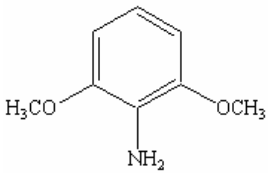
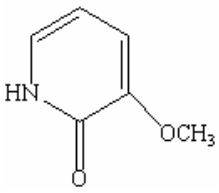
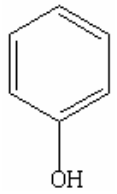
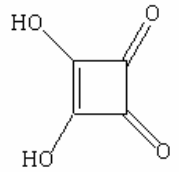
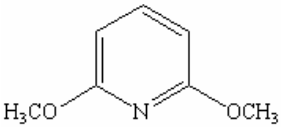
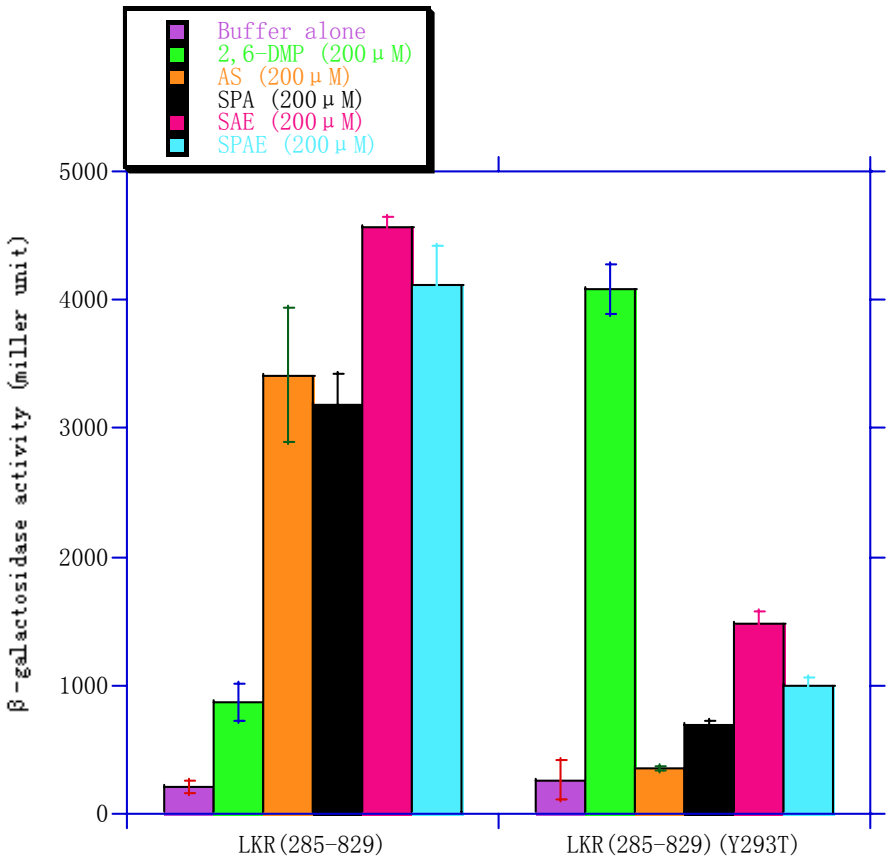
3, 5-Dimethoxyaniline 	3-methoxy pyridone 
phenol 	squaric acid 
3,5-dimethoxypyridine 	

Fig 4.10 Y293T directs specificity. Derivatives of *A. tumefaciens* A136/pRG109 carrying indicated plasmids pYW34 and pFQ282 were cultured in induction medium with either buffer alone or 200 μM inducers (2, 6-DMP, AS, SPA, SAE and SPAE) and assayed for the *vir* gene expression as described



(Melchers *et al*. 1989), while LKR(285-829)(Y293T) is turned off by these inducers relative to high activation by 2, 6-DMP. This result suggests that single mutation in the region aa288-aa294 prevents VirA/VirG activation by multiple phenolic compounds, in other word; residues in this region are involved in recognizing structurally diverse group of plant-derived phenolic compounds.

Discussion

In Chapter 3, Y293F/V765D was found to enhance the sensitivity and maximal response to AS. Although both genetic and physical evidence support an interaction between the linker and the receiver, the precise function remains in question. In this chapter, I have extended the study in the short region spanning aa285-293. I demonstrate that this region of the linker changes phenol specificity related with the para acetyl group and Y293T dramatically reduces the range of known *vir* inducers.

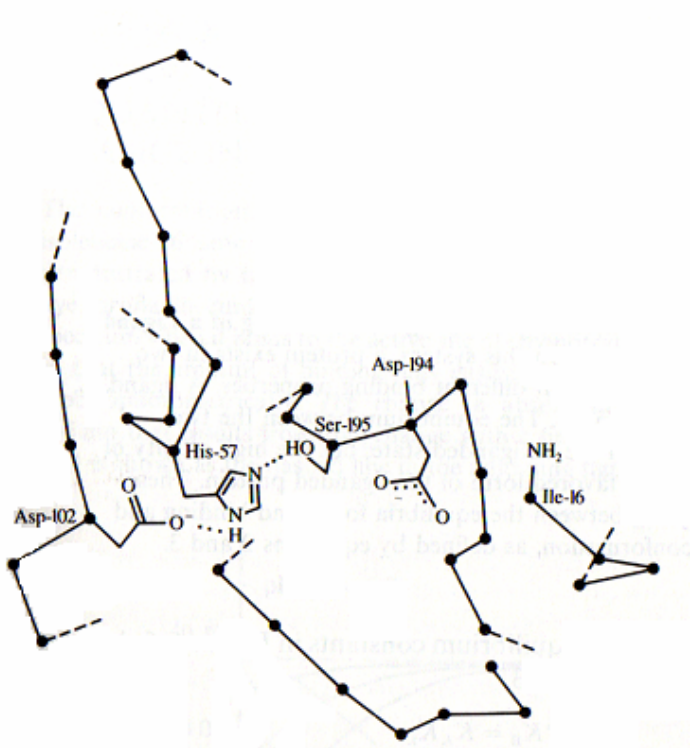
When aa285-293 was deleted, the VirA allele LKR(294-829) did not respond to AS. Protein expression and bacterial two-hybrid assay indicated that this short region affects neither VirA protein expression nor dimerization consistent with this region being involved in phenol recognition; specifically the para acetyl group of the inducers and the receiver does not contribute to the specificity. AS and 2, 6-DMP dose response assay indicates that the maximal activity change with different truncations is the determinant for phenol specificity change. Remarkably, single mutation Y293T prevents VirA/VirG activation by multiple phenolic compounds implying residues in this region are involved in recognizing structurally diverse group of plant-derived phenolic compounds.

According to the amino acid alignment and sequence analysis in the linker domain, aa288-aa293 exists in a loop connecting TM2 (aa279-aa287) to Helix-C, a predicted coiled coil starting from aa294. The simple model where this region forms a binding pocket is not consistent with truncation mutagenesis across the predicted loop. This loop may function distal to the binding pocket, regulating a conformational change of the phenol binding pocket. In the case of enzymatic catalysis by chymotrypsin, isoleucine-16 is not in the substrate binding pocket of the enzyme, but the ion pair linking isoleucine-16 and aspartate-194 provides an electrostatic interaction that is critical for maintaining the three-dimensional structure of the active enzyme. The active enzyme binds substrate at neutral pH, however, when the pH is increased and the isoleucyl-NH_3^+ group loses a proton, the ionic bond with apartate-194 is broken and the enzyme loses its ability to bind substrate as shown in Fig 4.10 (Almeida *et al* 1998). The aa288-293 loop may function in the same way.

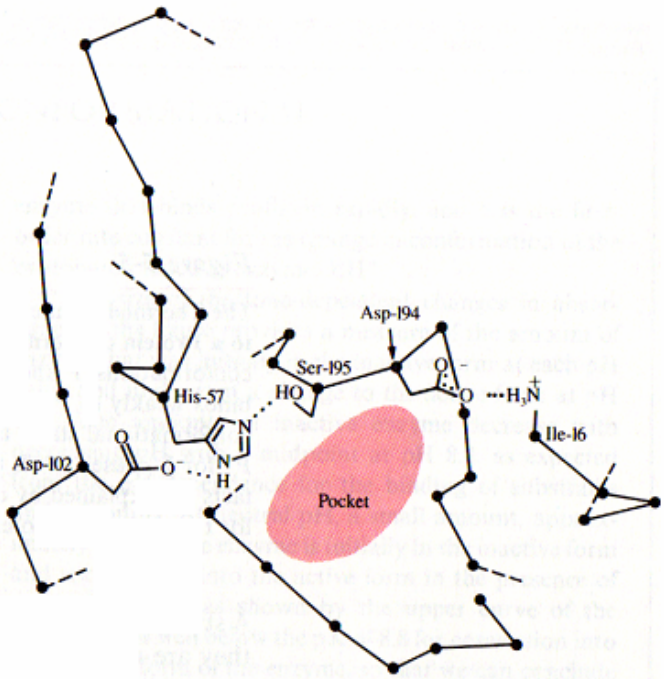
The interaction of the receiver with the linker remains a central question in this specificity and the overall mechanism of activation. For VirA/VirG two-component system activation, there are two critical steps, one is the autophosphrylation of His474 in the kinase, and the other is the phospho-transfer from His474 to the response regulator VirG. In Chapter 3, LKR(285-829)(V765D) is not active and LKR(285-829)(Y293F/V765D) significantly enhances the maximal response to AS in both P_{N25} driven and native promoter driven virG. Therefore, repression by the null mutation in the receiver does not alter phopho-transfer to VirG, but rather affect the rate of autophosphrylation of His474 through the interaction between the linker and the receiver. Since the receiver can interact with the kinase and the linker domains, is it possible that

the receiver interacts with VirG? Does the interaction between the receiver and VirG affect the rate of phospho-transfer? In Chapter 5, I studied the relationship between the VirA receiver and VirG.

Fig 4.11 Diagram of the substrate binding pocket change of chymotrypsin at different pHs. At high pH, the β-carboxylate group of aspartate-194 blocks the specificity pocket of chymotrypsin. At neutral pH, the protonated isoleucine-16 amino group forms an ion pair with the aspartate carboxylate group, which opens the pocket and allows the hydrophobic side chain of specific substrates to bind (Almeida *et al* 1998).



pH 10 (inactive)



pH 7 (active)

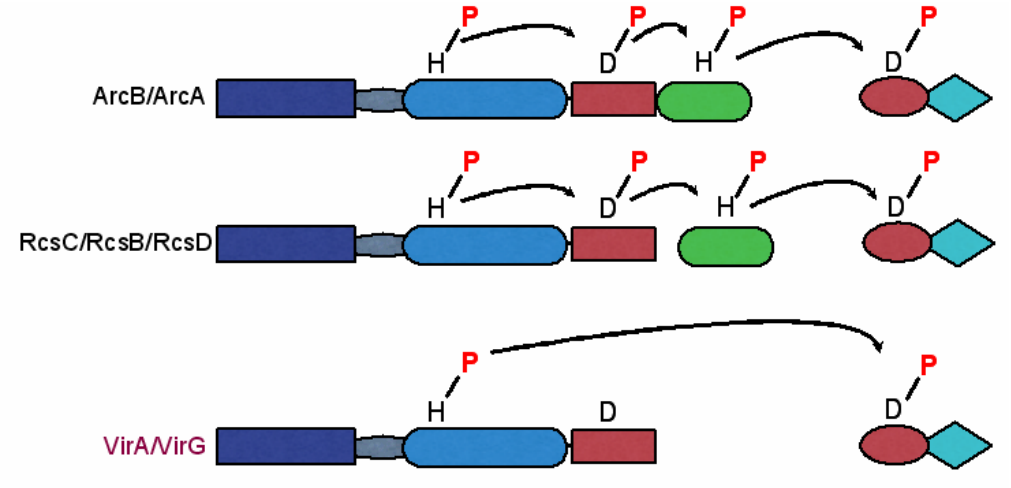
CHAPTER 5

The Receiver Domain of *Agrobacterium tumefaciens* VirA Histidine Kinase Has an Activating Role in *vir* Gene Expression

Introduction

In Chapter 3, evidence is presented for the specific interaction between the VirA linker and receiver domains which can decrease the rate of autophosphorylation of His474. A question was raised that does it regulate downstream signal events such as phospho-transfer to the response regulator VirG since it regulates upstream signal events through the linker? Generally, in two-component system, hybrid kinases, as shown in Fig 5.1, carry an additional C-terminal receiver domain whose sequence is homologous to the receiver domain of the response regulator. This additional receiver is characteristic of the conserved aspartate corresponding to that which becomes phosphorylated on the response regulator. In some cases, this aspartate has been shown to be essential for activation of the cognate response regulator in conjunction with HPT (Histidine-containing Phospho-Transfer) elements that intervene between the kinase and the response regulator, as in the His-Asp-His-Asp phospho-relay system (Georgellis *et al*. 1997; Takeda *et al*. 2001; Uhl *et al*. 1996). The HPT element may exist as a domain on the histidine kinase, such as ArcB in the ArcB/ArcA two-component system, and are located just after the receiver domain or as a separate protein such as RcsB in the RcsC/RcsB/RcsD two-component

Fig 5.1 Schematic representation of hybrid histidine kinase. Three different kinds of hybrid histidine kinases: one has a HPT domain located just after receiver domain (such as ArcB); one has a separate HPT domain (such as RcsB); the other has a receiver domain without HPT domain (such as VirA). Dark blue, input domain; blue, kinase domain; brown, receiver domain; green, HPT domain.



system. However, a survey of 156 complete microbial genomes indicated that hybrid kinases are almost five times as prevalent as identifiable HPT sequences (Zhang *et al*. 2005). This discrepancy may be due to limited sequence conservation among HPT domains, but another possibility is that the receiver domains of hybrid kinases have evolved diverse functions that do not involve an extended phospho-relay. One indication of such diverse functions comes from the study of "psuedo-receiver" domains. Pseudo-receiver domains are similar in sequence to the receiver domains of typical response regulators, but do not carry the conserved aspartate. Experimental evidence suggests that some pseudo-receivers act as localization or attachment elements (Zhang *et al*. 2002; O'Hara *et al*. 1999; Williams *et al*. 2006).

The function of VirA's receiver appears to differ from those carried on some other hybrid kinases in a number of ways. First, VirA does not appear to become phosphorylated on D766, the residue analogous to Asp52 on the VirG receiver domain (Mukhopadyhay *et al*. 2004) nor even require the domain for activity (Pazour *et al*. 1991). Second, VirA does not include an HPT domain. While, I cannot exclude the possibility of a separate HPT protein assisting in transfer of the phosphate from VirA to VirG, there is no evidence for one. In fact, purified VirA rapidly phosphorylates purified VirG, suggesting that intervening phospho-relay proteins are not required for VirG activation (Jin *et al*. 1990). Finally, previous analyses of VirA demonstrated that removal of the receiver allowed *vir* gene expression in the absence of a phenolic inducer, provided sugar and acidic pH were present (Chang *et al*. 1992; Chang *et al*. 1996; Brencic *et al*. 2004; Gao *et al*. 2005) consistent with the evidence that the receiver has a repression on the VirA activation demonstrated in Chapter 3. These experiments led to the description of

the VirA receiver domain as an inhibitory element. However, in most cases, the receiver in hybrid kinases acts as a bridge for phospho-transfer to the response regulator. Although the VirA receiver domain seems to function differently, it is still interesting and necessary to find out the relationship between the receiver and the response regulator VirG.

In contrast to the studies described above, the experiments presented here show that VirA's receiver domain behaves as an enhancing element. In fact, response to the phenolic inducer, in the absence of sugar, required the receiver domain. However, our studies utilized cells expressing *virG* from its own promoter, whereas the earlier studies that described the VirA receiver domain as inhibitory, utilized strains expressing *virG* from a constitutive promoter. I repeated the earlier result and, additionally, found that overexpression of *virG* corrected the apparent null phenotype of VirA mutants that carried point mutations in their receiver domains. Furthermore, regulation of *virG* expression from the P_{N25} promoter (Wang *et al*. 2000), via the LacI repressor, demonstrated that the activity of a VirA receiver truncation mutant requires a significantly higher concentration of VirG than full-length VirA. Finally, a bacterial two-hybrid assay demonstrated that the VirA receiver segment could interact with the DNA-binding region of VirG. Thus, the VirA receiver may function as a recruitment and/or alignment factor to effectively increase the availability of VirG for phosphate transfer from VirA's kinase region to the VirG receiver domain, suggesting a role not previously considered for the receiver domains of hybrid kinases.

Materials and Methods

Bacterial Strains, Media and Growth Conditions. Bacterial strains and plasmids used in this study are listed in Table 5.1. *E. coli*. XL-blue (Stratagene) and SU202 (Dmitrova *et al*. 1998) were grown at 37ºC in LB medium supplemented with the suitable antibiotics. *A. tumefaciens* strains were cultured at 25ºC in MG/L or AB*I (Wise *et al*. 2006) with the appropriate antibiotics for plasmid maintenance.

Table 5.1 Bacterial strains and plasmids used in Chapter 5.

Strains	Relevant features	Reference
E. coli		
XL1-blue	*recA1 endA1 hsdR17 [F' proAB lacI*^{q}*Z M15 Tn10(tet*^{r}*)]*	Stratagene
SU202	Ps*ulA-lacZ* fusion with hybrid operator sites (op408/op+)	(Dmitrova *et al*. 1998)
A. tumefaciens		
A348-3	C58 background with pTiA6NC, *virA*::*kan*^{r}	(Lee *et al*. 1992)
A136	C58 background without pTi	(Watson *et al*. 1975)
Plasmids		
pRG109	IncP plasmid carrying P*virB-lacZ* fusion and	(Gao *et al*.

	P_{N25}-*virG*, *Spec*^{r}	2005)
pSW209Ω	IncP plasmid carrying P*virB-lacZ* fusion, *Spec*^{r}	(Lee *et al*. 1992)
pSR658	Expression vector for fusion to wild type *lexA* DNA-binding domain	(Daines *et al*. 2000)
pSR659	Expression vector for fusion to mutant *lexA* DNA-binding domain	(Daines *et al*. 2000)
PMal-c2x	Source of *lacI*^{q}	New England Biolabs
pYW15b	IncW/ColE expression vector with P_{N25} and 6X-His, *Carb*^{r}	(Wang *et al*. 2000)
pYW47	pYW15b carrying *virG*, (codons 2 to 241) *Carb*^{r}	(Wang *et al*. 2000)
pAW10	IncW/ColE shuttle vector, *Carb*^{r}	(Wise *et al*. 2005)
pAW19	pUC19 carrying wild type *virA*, *Amp*^{r}	(Wise *et al*. 2005)
pAW16	pAW10 carrying wild type *virA*, *Carb*^{r}	(Wise *et al*. 2005)
pAW82	pUC19 carrying *virA*(Δ707-829)	This study
pAW100	pRG109 carrying wild type *virA*, *Spec*^{r}	This study
pAW102	pAW10 carrying *virA*(Δ707-829), *Carb*^{r}	This study
pAW103	pYW47 carrying virA(Δ707-829), *Carb*^{r}	This study

pAW97	pAW10 carrying virA(Δ712-829), *Carb*^{r}	This study
pAW106	pAW10 carrying *lacI*^{q}, *Carb*^{r}	This study
pAW104	pYW15b carrying the reciever domain of virG (codons 2 to 120), Carb^r	This study
pAW107	pRG109 carrying *virA*(Δ707-829), *Spec*^{r}	This study
pFF5	pYW15b carrying P_{N25}-*virA*, *Carb*^{r}	This study
pJZ6	Derivative of pYW15b carrying the linker, kinase and receiver domains of *virA*, *Carb*^{R}	(Zhang 2000)
pQF63	Derivative of pFF5 with V765D mutation	This study
pQF64	Derivative of pFF5 with L770P mutation	This study
pQF79	Derivative of pYW15b carrying codons 132 to 240 of *virG*	This study
pQF365	pSR658 carrying the *virG* receiver domain (codons 1 to 120)	This study
pQF366	pSR658 carrying the *virA* receiver domain (codons 712 to 829)	This study
pQF367	pSR658 carrying the *virA* kinase domain (codons 425 to 711)	This study
pQF368	pSR659 carrying the *virG* DNA-binding domain (codons 131 to 241)	This study

Table 5.2 Primers used in Chapter 5.

Primer Name	Sequence	Characteristics
2985	GTGTCTCAGAGCTTGTGACCG	Sense
3500stop	CCCTTTCCACGCGGTGCCTACTAGCGGCCGA AAAAACTGTCTG	antiSense
DraIIIstop	GCTAGTAGG*CACCGCGTG*GAAACGGGGAG	Dra III & Sense
4120	ACAACAGCCGTCGTCCACAC	antiSense
lacIQKpn I	GATCATG*GGTACC*CGTGGCCAGGACC	Kpn I & Sense
LacIQHIII	GTGAGC*AAGCTT*ACATTAATTGCGTTGC	Hind III & antiSense
FFA	CTGGTCATGGACGACCAAGCG	V765D & Sense
FFB	CGCTTGGTCGTCCATGACCAG	V765D & antiSense
FFC	CAAGCGTCTCCTCCTGAAGAT	L770P & Sense
FFD	ATCTTCAGGAGGAGACGCTTG	L770P & antiSense
FFE	GCT*GAGCTC*GGAAACGGGGAGATTGTGGC	Sac I & Sense
FFF	GC*GGTACC*GCAACTCTACGTCTTGAT	Kpn I & antiSense
FFG	GC*CTCGAG*ATTCAGCTTCTTGAACTC	Xho I & Sense

Primer Name	Sequence	Characteristics
FFH	GG*GGTACC*CTAACGCGGTGCCTTATTGCG	Kpn I & antiSense
FFI	GC*GTCGAC*TTTTGTTTACTTGACTGGACAC	Sal I & Sense
FFJ	GC*AAGCTT*GGCTGCCATCGTCCCCCC	Hind III & antiSense
FFK	CG*GGATCC*GATTGGTTAGCGCGGCGT	BamH I & Sense
FFL	CCTACTCGC*GGTACC*GCAACTCTACGTCTTGA	Kpn I & antiSense

Plasmid constructions. Standard methods were used for DNA ligation, PCR and plasmid isolation and analysis (Ausubel *et al*. 1992). The sequences of primers used in this work are listed in Table 5.2. Sequence analysis confirmed the correctness of all PCR products. The *virA*Δ707-829 receiver deletion was constructed using sequential PCR (Ausubel *et al*. 1992). Primer 2985 was used with primer 3500stop and primer DraIIIstop was used with primer 4120 in two initial PCR reactions that used pAW19 (Wise *et al*. 2005) as template. A third PCR used the self-annealing products of the first two reactions to create a 1.1 kb PCR product, which was digested with SnaB I and Dra III and used to replace the wild type region in plasmid pAW19, thus creating pAW82 which carries two stop codons after the *virA* kinase domain, effectively removing the receiver domain. The 4.6 kb Kpn I fragments of pAW19 and pAW82 were cloned into pRG109 (Gao *et al*. 2005) to create, respectively, pAW100 and pAW109. In addition to wild type *virA* or *virA*ΔR, these plasmids carry P_{N25}-*virG* (Wang *et al*. 2000) and P_{virB}-*lacZ*. The $lacI^q$ gene

was amplified from pMAL-c2x (New England Biolabs) using PCR with primers lacIQKpn1 and lacIQHIII. Following digestion with Kpn I and Hind III, the PCR product was cloned into pAW10 (Wise *et al*. 2005) to create pAW106, an IncW plasmid which carries $lacI^{Q}$. Plasmids pQF63 ($virA_{V765D}$) and pQF64 ($virA_{L770P}$) were derived from pFF5 through site-directed mutagenesis. Primers FFA and FFB were used to change V765 to an aspartate (V765D) and primers FFC and FFD were used to create the L770P mutation using sequential PCR. Upstream and downstream primers used in construction of both mutations were FFK and FFL. The PCR fragments were inserted into pJZ6 (Zhang *et al*. 2000). The receiver portions of *virA* carrying the point mutations were then used to replace the wild type fragments in pFF5.

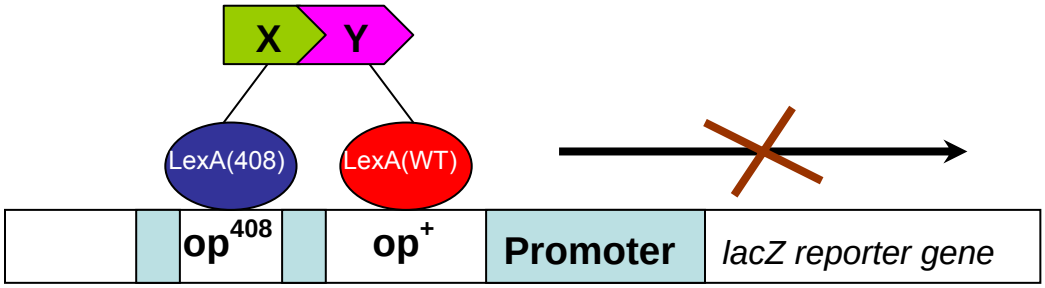
Plasmid pAW104 carries the P_{N25} promoter fused to the receiver domain (codons 2 to 120) of *virG.* The *virG* receiver domain was removed from pAW104 with Sac I and Kpn I digestion and cloned into pSR658 to create pQF365. The *virA* receiver domain (codons 712 to 829) was amplified using PCR with primers FFE and FFF using pFF5 as template. Following digestion with Sac I and Kpn I, the receiver fragment was cloned into pSR658 to create pQF366. Primers FFG and FFH were used to amplify the *virA* kinase region (codons 426 to 711, followed by a stop codon). Following digestion with Xho I and Kpn I, the kinase segment was inserted into pSR658 to make pQF367. Primers FFI and FFJ were used to amplify the DNA-binding domain (codons 132 to 240) of *virG* which was digested with Sal I and Hind III for cloning into pYW15b to create pQF79. The *virG* DNA-binding domain was removed from pQF79 with Sac I and Hind III for insertion into pSR659 to make pQF368. Immunoblot analysis confirmed the expression of the *lexA* fusions in pQF365, pQF366, pQF367, and pQF368.

***vir* Gene Induction Assays.** For *vir* gene induction, *A. tumefaciens* strains carrying a P_{virB}-*lacZ* fusion were grown overnight in MG/L, which was then used to inoculate AB*I containing glycerol or arabinose as a carbon source and the indicated amounts of the phenolic inducer acetosyringone (AS). Where IPTG was included, it was added in the amounts indicated. Following overnight incubation with shaking at 25ºC, β-galactosidase activity was determined by the method described by Miller (Miller *et al*. 1972).

Protein heterodimerization assay. The LexA-based bacterial protein interaction system has been described in Chapter 3 for homodimer assay. For heterodimer assay, there is a difference as described in Fig 5.2. *E. coli* strain SU202 carrying pQF368 was transformed with pQF365, pQF366, or pQF367. The protocol for checking the β-galactosidase activity and repression was the same as the one interpreted in Chapter 4.

Immunoblot Assay. A348-3 carrying pAW100 or pAW107 with either pAW10 or pAW106 were grown as described above for *vir* gene induction assays. Prior to the β-galactosidase assay, the OD600 of the cells samples was standardized. Equivalent volumes of whole cells were resuspended in sample buffer (3% SDS, 5% β-mercaptoethanol, 10% glycerol, pH 6.8) and heated for 5 minutes at 95°C before loading on an SDS polyacrylamide gel. VirG was visualized through Western blot analysis with RGS-His primary antibody (Qiagen), anti-mouse immunoglobulin secondary antibody and ECL Plus (Amersham Biosciences).

Fig 5.2 The lexA repressor-based bacterial two-hybrid system. For heterodimer interaction assay, X and Y represent two different proteins fused to wild type LexA DBD and modified LexA DBD. Interaction between X and Y produce a heterodimeric LexA repressor capable of binding to a hybrid operator site and repressing *lacZ* reporter gene expression.



Results

VirA receiver truncation alleles VirAΔ707-829 and VirAΔ712-829 have similar functions

Previously, the function of the VirA receiver domain was examined in strains with overexpressed VirG (Chang *et al*. 1992; Chang *et al*. 1996; Brencic *et al*. 2004; Gao *et al*. 2005). They truncated the receiver domain starting at the position aa712, just before receiver domain. Here, I truncated the receiver domain starting at aa707 to check whether the loop between kinase and receiver affects the function of receiver or not. *vir* gene expression was investigated at different conditions such as pH at 5.5 with glycerol or arabinose. Compared the activity between VirAΔ707-829 and VirAΔ712-829 shown in Fig 5.3, expression with and without AS were similar, although there is a subtle difference in the maximal activity indicating that the short region aa708-aa711 does not affect the function of VirA receiver domain, and VirA receiver domain still acts as an inhibitory element with constitutively expressed VirG.

The VirA receiver domain has an activating function.

I reexamined the effect of deleting the receiver domain on *vir* gene expression when *virG* remained under control of its natural promoter by following the expression of a *virB-lacZ* fusion (Fig 5.4). In the absence of sugar (Fig 5.4 A), VirAΔ707-829 (VirAΔR) behaved as a null mutant even at a relatively high concentration (300 μM) of the phenolic inducer. In contrast, when constitutively expressed *virG* (P_{N25}-*virG*) was included on the same plasmid, *virB-lacZ* expression was activated at lower concentrations of AS and

Fig 5.3 VirAΔ707-829 and VirAΔ712-829 have similar functions. Derivatives of *A. tumefaciens* A136/pRG109 carrying indicated plasmids pAW16, pAW102 and pAW97 were cultured in induction medium with 300μM AS and pH5.5 with different sugars and pH conditions as shown in the figure and assayed for the *vir* gene expression as described.

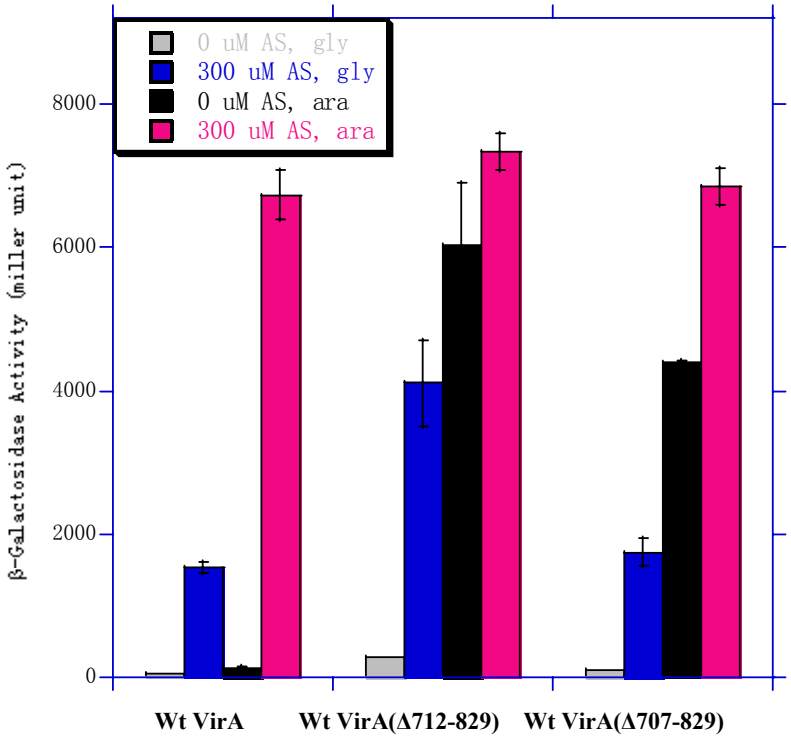
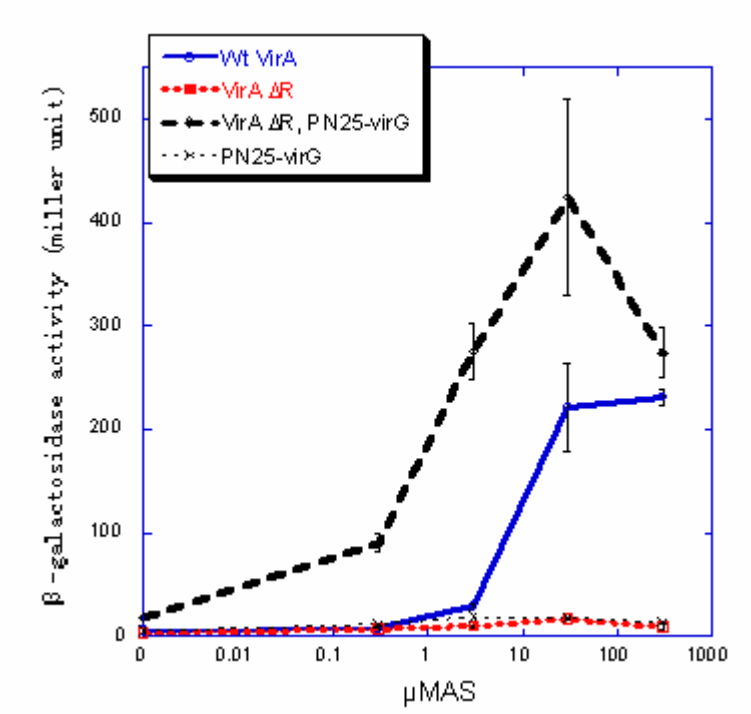
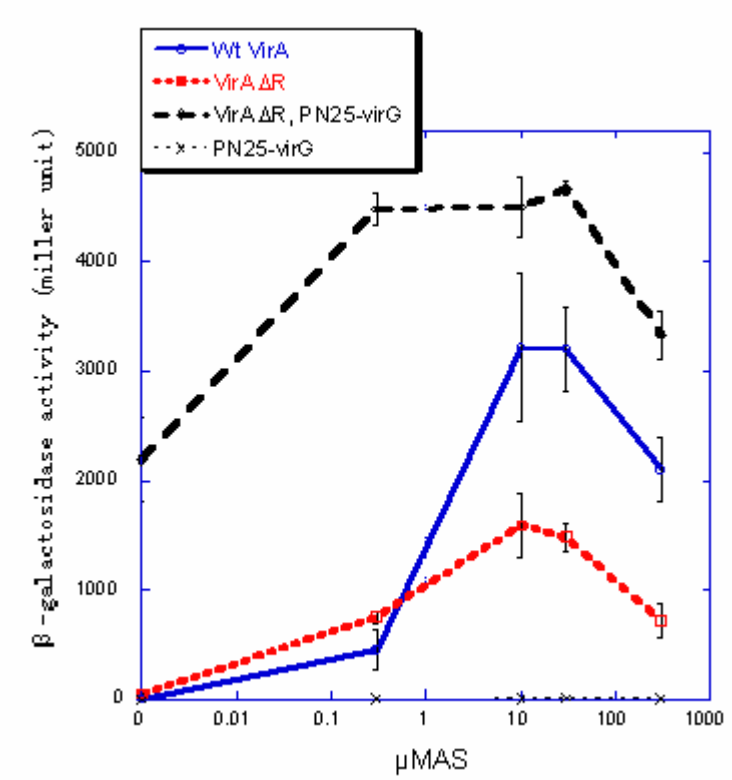


Fig 5.4 Removal of the VirA receiver domain results in defective signal-transducing activity when *virG* is expressed from its native promoter. Strain A348-3(Δ*virA*)/pSW209Ω (*virB-lacZ*) carried indicated plasmids pAW16, pAW102, pAW103 and pYW47 were cultured in AB*I medium with different concentrations of AS and sugars and pH5.5 and assayed for the *vir* gene expression as described. (A) Cells were grown with glycerol as the carbon source. (B) Cells were grown with arabinose as the carbon source. (C) Cells were grown with arabinose.

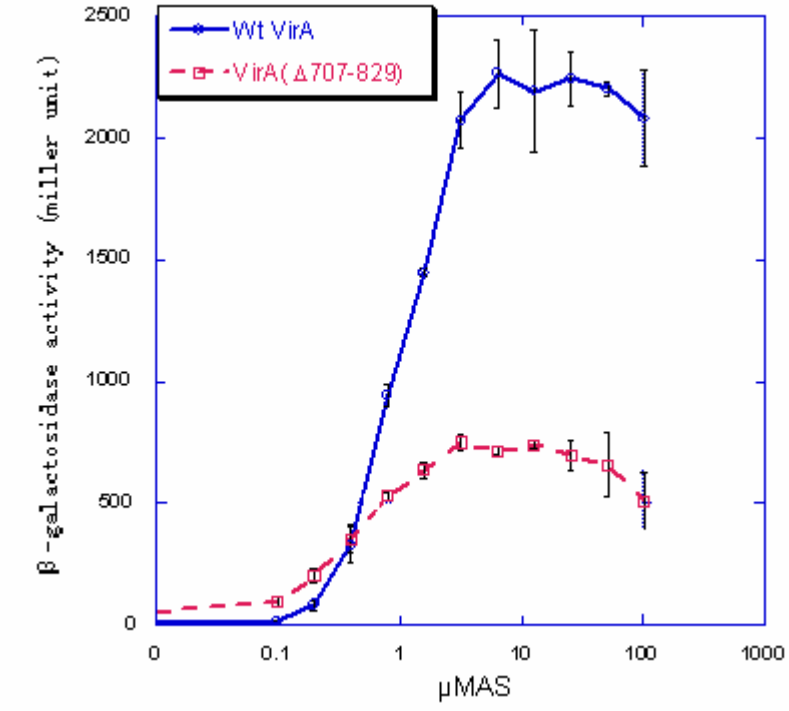
(A)



(B)


Wt VirA
VirA ΔR
VirA ΔR, PN25-virG
PN25-virG
β-galactosidase activity (miller unit)
5000
4000
3000
2000
1000
0
0
0.01
0.1
1
10
100
1000
μMAS

(C)


Wt VirA
VirA(Δ707-829)
β-galactosidase activity (miller unit)
2500
2000
1500
1000
500
0
0
0.1
1
10
100
1000
μMAS

reached a higher maximum activity than that achieved when *virG* was expressed from its native promoter. When sugar was included in the induction medium (Fig 5.4B), VirAΔR was capable of activating VirG, but the maximum level of *vir* gene expression was low compared to that determined by wild type VirA. In this medium, constitutive expression of *virG* strongly enhanced the activity of VirAΔR and allowed it to function without the phenolic inducer. The results derived from cells that constitutively express *virG* in the presence of VirAΔR agree with the observations of others which initially led to the characterization of the receiver domain of VirA as an inhibitory element (Chang *et al*. 1992; Chang *et al*. 1996; Brencic *et al*. 2004; Gao *et al*. 2005). More precise acetosyringone concentration dependent assays indicated that similar doses of the phenolic inducer (0.8 to 1.6 μM) resulted in 1/2 maximum activity for both VirA and VirAΔR, indicating that the mutant retains sensitivity to the inducer (Fig 5.4C). However, at very low concentrations of acetosyringone (less than 0.5 μM); I consistently observed that *vir* gene expression mediated by the mutant was slightly higher than that seen for wild type VirA (Fig 5.4C). Thus, the receiver domain seems to integrate divergent functions and may activate or inhibit different aspects of the protein's signal-transducing ability.

VirAΔR requires a higher concentration of VirG than wild type VirA in order to activate *vir* gene expression.

I used *virG* expressed from the P_{N25} promoter and regulated by the LacI repressor to examine the relationship between VirAΔR and the amount of VirG in the cells. Plasmid derivatives of pRG109 (Gao *et al*. 2005) which carry P_{N25}-*virG*, a *virB*-*lacZ* fusion and

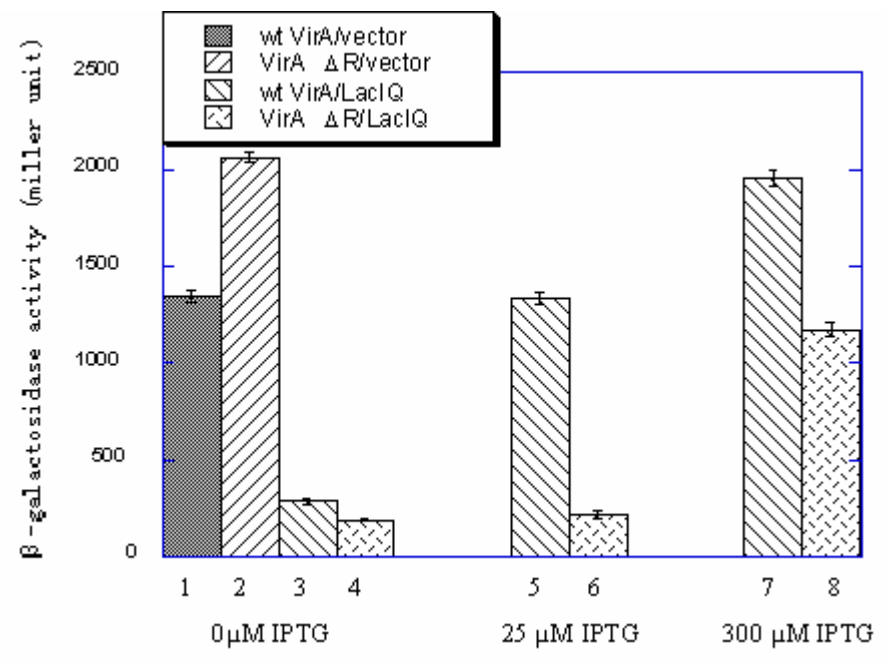
either wild type *virA* (pAW100) or *virA*Δ707-829 (pAW107) were introduced into strain A136. A136 does not contain the Ti plasmid and, therefore, does not contain wild type *virA* or *virG*. Transcription from P_{N25} (which carries two copies of the *lac* operator) is normally constitutive in *A. tumefaciens* lacking the LacI repressor. To control VirG expression, I added pAW106, a derivative of pAW10 that carries the $lacI^Q$ gene.

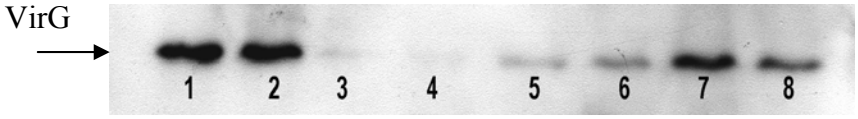
Cells that did not carry $lacI^Q$ had high levels of VirG, and as expected, the receiver truncation mutant expressed the *virB-lacZ* fusion at a higher level (Fig 5.5 lanes1 and 2). The presence of LacI in the cell significantly reduced the levels of both VirG and *vir* gene expression independent of the version of VirA without IPTG (lanes3 and 4). Induction of the cells that contained LacI, in the presence of 25 μM IPTG, modestly increased the cellular VirG content and significantly increased *vir* gene expression mediated by wild type VirA. *vir* gene expression that depended on VirAΔR remained suppressed (lanes5 and 6). Culturing these strains in the presence of 300 μM IPTG further increased VirG levels and *vir* gene expression for both strains (lanes7 and 8). This result suggests that the ability of VirAΔR to activate VirG is relatively poor, but can be corrected by sufficiently overexpressing of VirG.

Point mutations in the VirA receiver domain can be corrected by overexpression of VirG.

V765D and L770P were isolated following random mutagenesis of the VirA receiver domain of LKR (a gene fragment that includes the linker, kinase and receiver domains of VirA) and characterized as point mutations that produced a null phenotype. Because sugar and constitutively expressed VirG strongly enhanced the signal-

Fig 5.5 VirAΔR requires a higher cellular content of VirG than wild type VirA, in order to activate *vir* gene expression. A136 carrying derivatives of pRG109 which contains P_{N25}-*virG*, *PvirB-lacZ*, and either wild type *virA* (pAW100: entries 1, 3, 5, and 7) or *virA*ΔR (pAW107: entries 2, 4, 6, and 8). Entries 1 and 2 indicate cells that carried the empty pAW10 vector, while entries 3 to 8 indicate the presence of $lacI^Q$ on pAW106 (a derivative of pAW10). Cells were grown in AB*I induction medium with glycerol as the carbon source and 300 μM of acetosyringone. IPTG was added at the indicated amounts of 0, 25 or 300 μM. The β-galactosidase assay indicates the expression of the *virB-lacZ* fusion. The coordinated western blot indicates the amount of VirG in the cells used in the β-galactosidase assay.

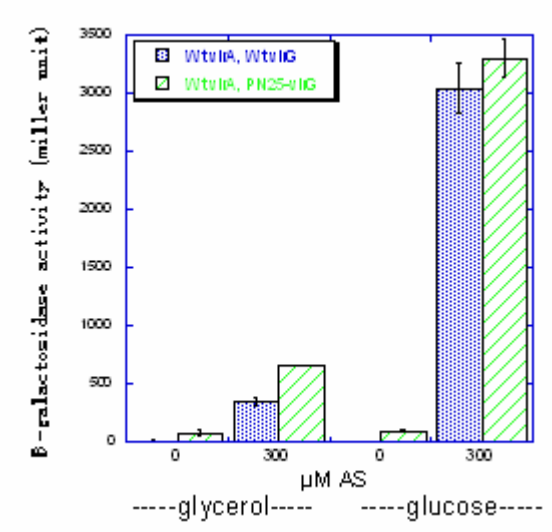

wt VirA/vector
VirA ΔR/vector
wt VirA/LaclQ
VirA ΔR/LaclQ
β-galactosidase activity (miller unit)
2500
2000
1500
1000
500
0
1 2 3 4 5 6 7 8
0μM IPTG
25 μM IPTG
300 μM IPTG


VirG
1 2 3 4 5 6 7 8

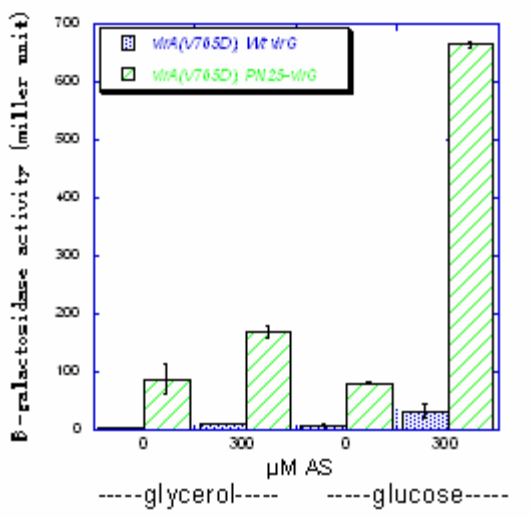
transducing activity of VirAΔR, I asked if these conditions would affect the phenotype of the two receiver mutants. Since transcription of *virA* and *virG* are auto-regulated when expressed from their native promoter systems (Winans *et al*. 1998), I placed *virA* under control of P_{N25} to bypass the effects of autoregulation on the amounts of VirA in the cells. The V765D and L770P receiver point mutations were each placed into full-length P_{N25}-*virA*, and the ability of the mutants to activate *vir* gene expression was compared with that of wild type VirA, in the presence and absence of P_{N25}-*virG*. The signal-transducing activity of wild type VirA was enhanced about two-fold by constitutive expression of *virG* in the glycerol medium with 300 μM AS (Fig 5.6A). However, the effect of sugar on the activity of wild type VirA was much stronger than the effect of constitutively expressed VirG. In contrast, sugar had little effect on the activity of $VirA_{V765D}$ (Fig 5.6B) or $VirA_{L770P}$ (Fig 5.6C), when only wild type *virG* was present. While the receiver domain mutations may have destroyed VirA's signal transducing function, overexpression of VirG from P_{N25} strongly enhanced the activity of both mutants, effectively reversing their null phenotypes. This effect was most pronounced for $VirA_{L770P}$ where, with 300 μM AS, constitutive expression of VirG increased activity, relative to that depending on native VirG, more than fifty-fold in the glycerol induction medium and more than sixty-fold when sugar was present. Thus, the receiver mutations did not inactivate VirA, but diminished its ability to stimulate VirG expression. The defect in activation of VirG was largely overcome when the concentration of that protein was increased through constitutive expression.

Fig 5.6 Constitutive expression of *virG* corrects the null phenotype of receiver point mutations. The signal-transducing activity of wild type VirA, $VirA_{V765D}$ and $VirA_{L770P}$ in A348-3 (Δ*virA*)/pSW209Ω (*virB-lacZ*) is compared with that in A348-3/pRG109 (P_{N25}-*virG*, *virB-lacZ*). All *virA* alleles are constitutively expressed from P_{N25}. As the legend in panel A indicates, green columns represent cells that carry constitutively expressed *virG*, while the blue columns indicate that *virG* is expressed only from its native promoter. Cells are grown in AB*I medium with 0 or 300 μM AS and either glycerol or glucose, as indicated. (A) pFF5 (wild type *virA*), (B) pQF63 ($virA_{V765D}$), (C) pQF64 ($virA_{L770P}$).

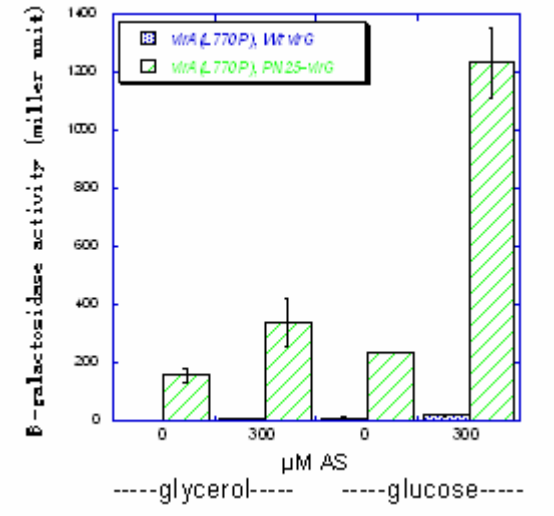
(A)


β-galactosidase activity (miller unit)
WtvirA, WtvirG
WtvirA, PN25-virG
0
300
0
300
μM AS
-----glycerol-----
-----glucose-----

(B)


β-galactosidase activity (miller unit)
virA(V765D), WtvirG
virA(V765D), PN25-virG
0
300
0
300
μM AS
-----glycerol-----
-----glucose-----

(C)

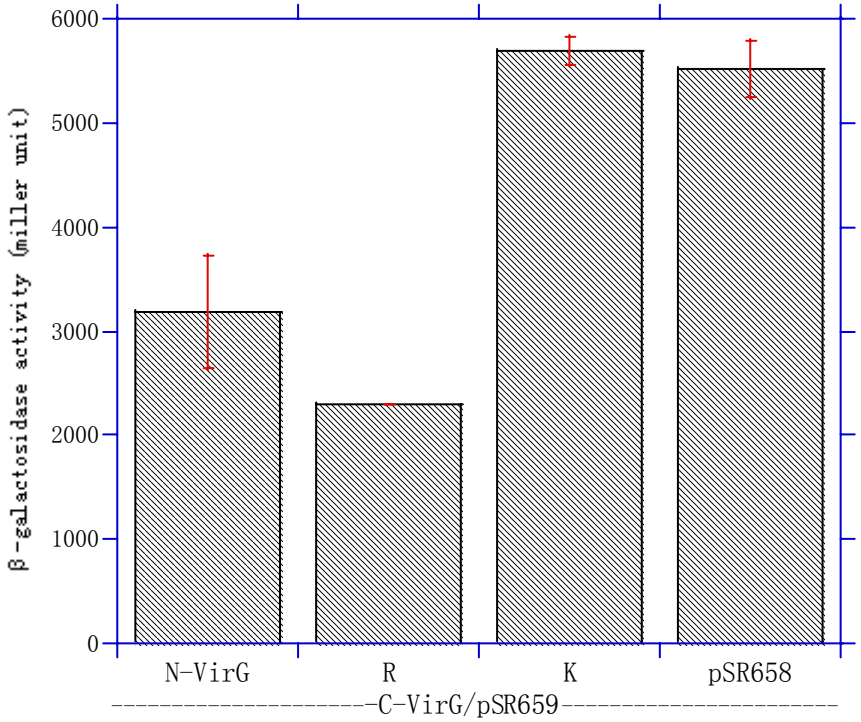

β-galactosidase activity (miller unit)
virA(L770P), WtvirG
virA(L770P), PN25-virG
0
300
0
300
μM AS
-----glycerol-----
-----glucose-----

The DNA-binding domain of VirG interacts with the receiver domain of VirA.

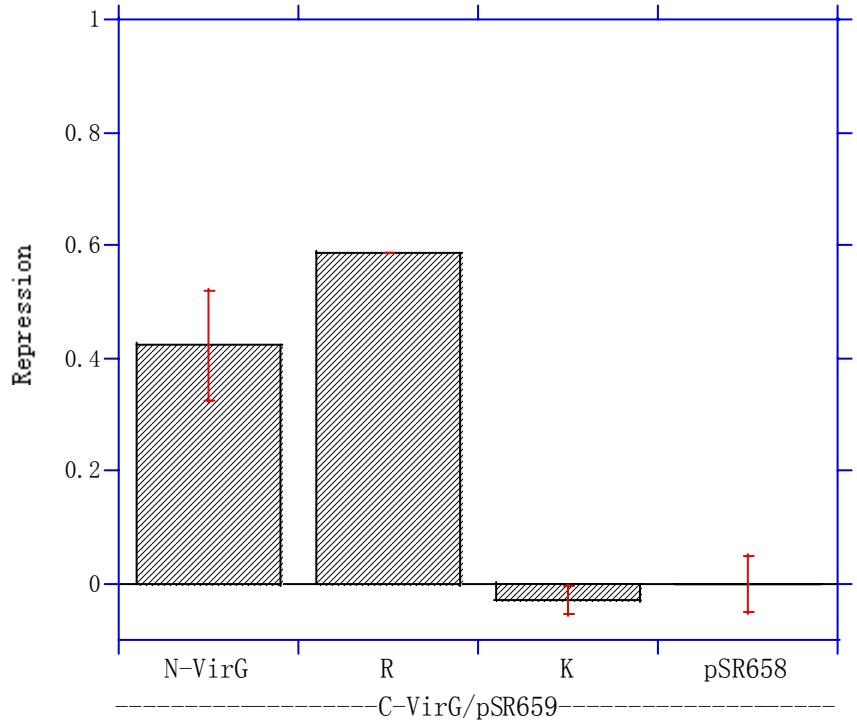
Structural studies of some response regulators indicate that their activity may be regulated through extensive interdomain contacts between the effector (often a DNA-binding) domain and the receiver region that contains the phosphorylatable aspartate (Baikalov *et al*. 1996; Djordjevic *et al*. 1998; Robinson *et al*. 2003). According to a FASTA analysis, the sequence homology between the receiver domains of VirA and VirG is 24% identity and 55% similarity. The homology between the two receiver domains suggests that, if the receiver domain of VirG interacts with its effector domain, then so might the receiver domain of VirA. I used a bacterial two-hybrid system based on the LexA repressor (Dmitrova *et al*. 1998; Daines *et al*. 2000) to analyze interactions between the VirG DNA-binding domain and the VirA and VirG receiver domains. For this experiment, the receiver portions of VirA and VirG were individually fused to the DNA-binding region of wild type *lexA* carried in pSR658 (Daines *et al*. 2000), while the DNA-binding domain of *virG* was fused to a mutated version of the DNA-binding region of *lexA* carried in pSR659 (Daines *et al*. 2000). Heterodimer formation that brings together the mutant and wild type DNA-binding portions of LexA represses transcription of a LexA regulated P_{sulA}-*lacZ* fusion. The presence of the empty "bait" and "prey" vectors sets the unrepressed level of β-galactosidase activity in one strain, while a decline in β-galactosidase activity in cells that express the LexA fusion to VirG's effector domain indicates heterodimer formation between that protein and a LexA-receiver fusion expressed from pSR658. Fig 5.7 represents the data as repression of the P_{sulA}-*lacZ* fusion. The results of the two-hybrid experiment indicate that the DNA-binding domain of VirG can interact with the receiver domain of VirA, as well as with its own receiver fragment.

Fig 5.7 The VirA and VirG receiver domains interact with the DNA-binding domain of VirG. *E. coli* SU202 (P_{sulA}-*lacZ*, op408/op+) carrying the pSR658 and pSR659 vectors (1st column), or pQF368 (pSR659 + the DNA-binding domain of VirG), and, as indicated, either the VirG receiver (2nd column) or VirA receiver (3rd column) or the VirA kinase (4th column) on pSR658. Repression of P_{sulA}-*lacZ* fusion expression is represented. (C) Western blot analysis with anti-lexA antibody. lane1, N-VirG/pSR658+C-VirG/pSR659 (for N-VirG/pSR658: protein size: 23.4 kDa); lane2, R(711-829)/pSR658+C-VirG/pSR659 (for R/pSR658: protein size: 21.6 kDa); lane3, K(426-711)/pSR658+C-VirG/pSR659 (for K(426-711)/pSR658, protein size : 40.7 kDa) lane4: pSR658+pSR659.

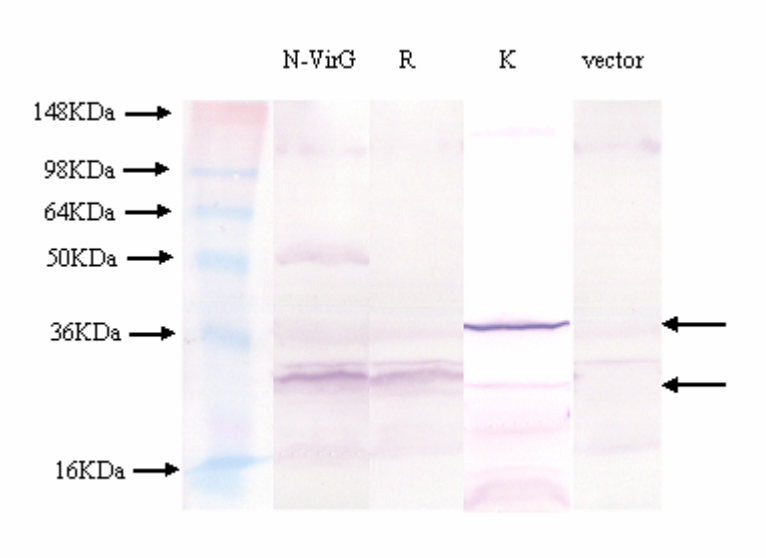
(A)


β-galactosidase activity (miller unit)
6000
5000
4000
3000
2000
1000
0
N-VirG
R
K
pSR658
C-VirG/pSR659

(B)


Repression
1
0.8
0.6
0.4
0.2
0
N-VirG
R
K
pSR658
C-VirG/pSR659

(C)



The VirA kinase region, included as a negative control, did not interact with VirG's DNA-binding domain. Protein has been expressed for each sample by checking the total protein expression using Western blot (Fig 5.7C) and a similar assay was used to show that the VirA receiver did not interact with the VirG receiver (data not shown).

Discussion

In this chapter, I have demonstrated that the receiver domain of VirA is an activating factor for *vir* gene expression in *A. tumefaciens*. When VirG was expressed from its native promoter, a form of VirA that lacked the receiver domain, it demonstrated an absolute requirement for the auxiliary sugar signal. The signal-transducing activity of VirAΔR in the presence of sugar and a phenolic inducer was generally inferior; maintaining a maximum activity between 30% and 50% that of full-length VirA. In contrast, if the cells included constitutively expressed VirG, VirAΔR no longer required sugar, but if it was present, the protein was active in the absence of a phenolic inducer. This latter result is similar to the findings that led to the characterization of the VirA receiver domain as inhibitory (Chang *et al*. 1992; Chang *et al*. 1996; Brencic *et al*. 2004; Gao *et al*. 2005). The action of the receiver, in keeping VirA inactive in the absence of a phenolic inducer, may be biologically significant because the enzymatic activity that produces cinnamic acid, a precursor of acetosyringone and other phenolics, peaks and then declines within a day or two after wounding (Kahl *et al*. 1982). If the levels of VirG that follow induction are still high, the VirA receiver could be important in shutting off

vir gene expression.

Using a system that allowed us to control the amount of VirG in the cell, I found that VirAΔR actually required a higher cellular content of VirG in order to activate *vir* gene expression. Thus, the abundance of VirG available when VirG is constitutively expressed apparently facilitates effective interaction between VirAΔR and VirG. The positive effect of overexpressed VirG may be due, in part, to obviating the need for auto-regulated induction of VirG. Normally, inducing conditions strongly up-regulate expression of both *virA* and *virG* expression in a VirA/VirG dependent manner (Winans *et al*. 1988). In addition, a basal level of VirG is expressed from a second promoter independent of VirA or VirG (Winans *et al*. 1990). *vir* gene expression, then, requires a form of VirA capable of interacting with VirG when the concentration of that protein is at the low basal level.

Versions of VirA that carried receiver point mutations retained their null phenotypes when constitutively expressed from P_{N25}, suggesting that even when the autoregulatory effect on the cellular content of $VirA_{V765D}$ and $VirA_{L770P}$ was bypassed, these proteins were unable to effectively interact with VirG when it was present at basal levels. Increasing the cells VirG content with a P_{N25}-*virG* construct allowed $VirA_{V765D}$ and $VirA_{L770P}$ to interact effectively with VirG, leading to *vir* gene expression.

Although the enhancing effect of the receiver domain was clear when *virG* was expressed from its native promoter, I also consistently noted a slight increase in *vir* gene expression in the absence of the receiver and at very low concentrations of the phenolic inducer. This observation may be reflective of the inhibitory effect of the receiver seen when *virG* is constitutively expressed (shown in Chapter 3). Interpretation of these

seemingly contradictory observations is aided by an analysis of *A. rhizogenes* VirA (Endoh *et al*. 1993). An *in vitro* phosphorylation study used a version of the protein that lacked the periplasmic and linker regions. The authors found that an additional deletion within the receiver domain increased the protein's rate of auto-phosphorylation. Faster autophosphorylation might be expected to increase the protein's signal-transducing activity. However, it was further shown that the same receiver deletion reduced the rate of phospho-transfer to VirG (Ausubel *et al*. 1992). This *in vitro* experiment agrees with our observations that the receiver domain encompasses both activating and inhibitory elements. Our finding that phospho-transfer can be facilitated by increasing the cellular content of VirG (Fig 5.5) suggests that a concomitant ability to auto-phosphorylate might Ill increase the activity of VirAΔR relative to full-length VirA when VirG is constitutively expressed. However, when VirG is expressed from its native promoter, wild type VirA's proficiency in phospho-transfer might overcome the VirAΔR advantage in auto-phosphorylation as levels of inducer increase.

The two-hybrid experiment demonstrated that VirG's DNA-binding domain could interact with its own receiver and that of VirA. The first result is in line with structural studies that have revealed extensive interactions between the receiver and effector domains of other response regulators (Baikalov *et al*. 1996; Djordjevic *et al*. 1998; Robinson *et al*. 2003). This interaction appears to have a regulatory effect that controls the activity of the effector domain under non-inducing conditions. In the case of the NarL response regulator, phosphorylation of the receiver domain has been shown to bring about domain separation, which makes the protein's DNA-binding domain available for binding at promoter sites in its regulon (Zhang *et al*. 2003).

The interaction between the VirA receiver and the effector domain of VirG suggests an explanation for the observation that deletion of the VirA receiver domain diminishes *vir* gene expression unless the cellular content of VirG is particularly high. VirA is a membrane-bound protein, while VirG is cytosolic. Activation of VirG likely requires a fairly specific alignment between the VirA kinase region and the VirG receiver. Thus, molecular collisions through Brownian motion may not achieve phospho-transfer at a rate that allows rapid induction of the two proteins through auto-regulation. Some sensor proteins have been shown to carry "recruiting" or "localization" elements that increase the probability of interaction with their cognate regulatory factors (Williams *et al*. 2002; O'Hara *et al*.1999; Zhang *et al*. 2006; Loplan *et al*. 2003; Andrade *et al*. 2006). I propose that attachment of the VirA receiver to the VirG effector domain increases the occurrence of productive interactions between the VirA kinase region and VirG's receiver domain, thus promoting the phospho-transfer event. Moreover, this may be an important mechanism to consider in evaluating the activities of other hybrid histidine kinases, particularly those that do not appear to contain an HPT domain.

CHAPTER 6

SUMMARY

Cells utilize signal transduction networks to properly adapt to external stimuli, and to respond appropriately to the environment. It is essential for the organism to perceive and distinguish between various stimuli and transduce these input signals accurately to activate appropriate responses. In prokaryotes, fungi and plants, two-component regulatory systems composed of HK (histidine kinase) and RR (response regulator) have been frequently exploited to sense and adjust to environmental fluctuation. Since environmental signal detection and transduction are critical for survival of organisms, it is very important to understand how specific "information" (signals) are perceived, transferred and translated by two-component systems. In this study, the VirA/VirG two-component system has been investigated as a model for these robust and widely dispersed signal perceiving elements.

Signal perception and integration by the VirA/VirG two-component system is critical for the pathogenesis of *Agrobacterium*. Owing to the well characterized input signals, the understanding of signal sensing and transmission by the kinase sensor VirA is very likely to underpin and enrich the perspective of the general strategy for signal perception and transduction used by two-component systems. Of the three classes of signals (monosaccharide, acidic pH and phenolic compounds) sensed by the histidine sensor kinase VirA, the perception of monosaccharide and low pH is located in the

periplasmic domain while the sensing of phenolic compounds is assigned to the linker region connecting the membrane spanning helices to the kinase. At the time I began my study, two hypotheses, separate phenolic receptors and VirA as the direct sensor, existed. In the Chapter 2, the VirA/VirG system was transferred into the heterologous *E. coli* host and I found that the ability for inducing and inhibiting *vir* induction both in *E. coli* and in *A. tumefaciens* was similar; strongly supporting VirA was both necessary and sufficient for phenols perception.

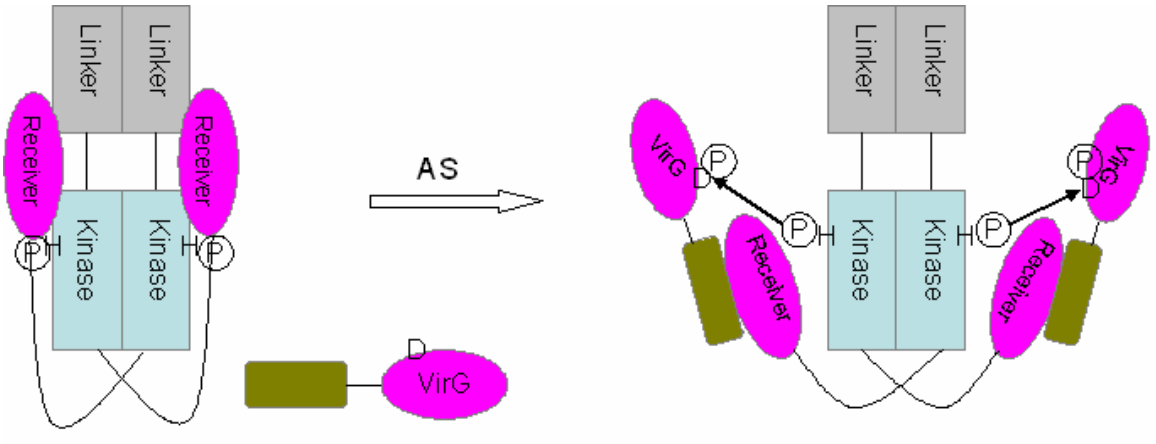
VirA has been dissected into four domains (Chang et al 1992), the periplasmic, linker, kinase and receiver. One striking feature, in contrast to most two-component signal modules, is that VirA carries an additional receiver at its C-terminus. Although this domain has been described as inhibitory, the actual function remains unclear. In the Chapter 3, two null mutations V765D and L770P are discovered in the receiver, and compensating alleles were found within the linker domain. The double substituents alleles, e.g. LKR(285-829)(Y293F/V765D), are particularly noteworthy. The dramatic activity of Y293F/V765D implicates an interaction between the linker and the receiver domain and physical evidence (crosslinking data) supports the existence of the interaction. Based on the previous evidence that the receiver functions as a repressor of VirA activation, I propose signal induction relieves the inhibition through dissociation of the linker/receiver interaction.

In most cases, the receiver in hybrid kinases acts as a bridge for phospho-transfer to the response regulator (Takeda et al. 2001). The VirA receiver domain then seems to function differently. Indeed, in Chapter 5, evidence is presented that the receiver functions as an enhancing element, possibly by increasing the availability of VirG for

phospho-transfer. For the VirA/VirG activation, there are two critical steps, autophosphorylation at His474 in the VirA kinase domain and phosphor-transfer from His474 to the response regulator VirG. Previous in vitro phosphorylation results suggest that the receiver decreases the rate of auto-phosphorylation while it increases the rate of phospho-transfer to VirG. The data of Chapter 3 and Chapter 5 lead to a model for the role of the receiver in the VirA/VirG activation shown in Fig 6.1. The receiver domain encompasses both activating and inhibitory elements. With signal induction, the repression is relieved through dissociation with the linker which makes the receiver to interact with the DNA binding domain of VirG for facilitating the phospho-transfer from VirA kinase to the receiver domain of VirG.

Even with this mechanistic insight, how VirA detects multiple phenolic compounds remains unclear. In Chapter 3, LKR(285-829)(Y293F) dramatically increases the sensitivity and LKR(285-829)(Y293F/V765D) significantly enhances the sensitivity and maximal response to AS. Clearly, aa293 is critical for signal regulation in VirA/VirG two-component system. This residue is located in the region predicted to be a loop region including aa285-293. In Chapter 4, the short region aa288-293 in the linker is found to regulate phenols specificity. The results of stepwise truncation of this short region suggest that although aa288-293 is not located in the binding pocket, it regulates the conformational change of the binding pocket for phenols induction. Most importantly, single mutations such as Y293T in this region greatly reduce the range of known *vir* inducers suggesting this region has an important biological function for *Agrobacterium tumefaciens* as a multi-host pathogen. These alleles will now enable direct tests of the importance of responding to many different signals in multi-host pathogenesis.

Fig 6.1 Model for VirA/VirG two-component system activation



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