Plant Signaling & Behavior 8:10, e26784; October; © 2013 Landes Bioscience

The role of RAR1 in *Agrobacterium*-mediated plant transformation

Ajith Anand[†] and Kirankumar S Mysore*

Plant Biology Division; Samuel Roberts Noble Foundation; Ardmore, OK USA

[†]Current Address: DuPont Pioneer; Johnston, IA USA

Keywords: *Agrobacterium,* F-box proteins, plant transformation, SCF-E3 ligase, VIGS

RAR1 is identified as a critical protein involved in plant innate immunity. We investigated the role of RAR1 in *Agrobacterium*-mediated plant transformation based on the previous findings that accessory proteins associated with the E3 ligase complex such as SGT1, which tightly interacts with RAR1, play a role in the transformation process. *RAR1* gene silencing in *Nicotiana benthamiana* and *Arabidopsis rar1* mutant analyses suggested that *RAR1* is required for early stages of *Agrobacterium*-mediated plant transformation. This finding further illustrates that RAR1, along with SGT1, that serve as a HSP90 co-chaperone is important for *Agrobacterium*-mediated plant transformation.

Introduction

One of the critical steps in *Agrobacterium*-mediated genetic transformation involves the uncoating of the transferred DNA (T-DNA) from its cognate protein complex before integration. This step in the transformation process is not clearly understood, even though the involvement of the host ubiquitin-proteosome system (UPS) has been suggested.[1] One of the most extensively studied UPS in plants includes the SKP1/Cullin/F-box (SCF) E3 ubiquitin ligase complex which is induced upon *Agrobacterium tumefaciens* infection[2] and mediates degradation of the cognate proteins either through the induction of a plant specific F-box protein VBF[3] or by the *Agrobacterium* F-box protein VirF, which is exported into the host cell.[4]

The plant SCF complex mediates polyubiquitination of host and foreign proteins for degradation, thus controlling a plethora of biological process within the cell. Recent studies have shown differential expression of the UPS-associated genes upon *Agrobacterium* inoculation,[2] some of which likely affects *Agrobacterium* infection.[2,5] In addition, accessory proteins such as SGT1 (suppressor of the G2 allele of skp1) which loosely associate with SCF E3 ligases but tightly interacts with RAR1, a protein required for *R*-gene resistance to powdery mildew in barley,[6,7] also affect *Agrobacterium* infection.[2] Both RAR1 and SGT1 were identified as components of plant innate immunity.[8-12] Here we report the role of RAR1 in early stages of *Agrobacterium*-mediated plant transformation.

Results and Discussion

Gene silencing of *RAR1* attenuates *Agrobacterium*-mediated plant transformation

Using a virus-induced gene silencing (VIGS)-based approach, we previously showed[2] that Nb*RAR1* silenced *Nicotiana benthamiana* plants produced significantly smaller and fewer tumors when compared with non-silenced control plants upon inoculation with an oncogenic strain, *A. tumefaciens* A348.[13] For further characterizing the role of RAR1 in plant transformation, we performed additional experiments. Relying on the transient expression assay of β-glucuronidase (GUS) gene described by Anand et al.,[14] we observed moderate reduction in 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc) staining and GUS activity in the leaf disks derived from Nb*RAR1* silenced *N. benthamiana* plants as compared with leaf disks derived from control (TRV::*GFP* inoculated plants) or wild-type plants at early stages of transformation (4 and 7 dpi; **Figure 1A**). Based on the above data we suggest that gene silencing of *RAR1* results in reduced transient T-DNA gene expression. In addition to transient transformation, stable transformation experiments were performed to select glufosinate ammonium (GF)-resistant calli as described.[14] Silencing of Nb*RAR1* produced fewer GF-resistant calli (70.6%), when compared with wild-type or controls (85.6% and 91.1%, respectively, **Figure 1B**). When cultured on a non-selective callus-inducing medium, leaf disks from Nb*RAR1* silenced and control *N. benthamiana* plants produced calli at equal efficiency (data not shown). These data

*Correspondence to: Kirankumar S Mysore; Email: ksmysore@noble.org
Submitted: 08/28/2013; Accepted: 10/11/2013
Citation: Anand A, Mysore KS. The role of RAR1 in Agrobacterium-mediated plant transformation. Plant Signaling & Behavior 2013; 8:e26784; PMID: 24168895; http://dx.doi.org/10.4161/psb.26784

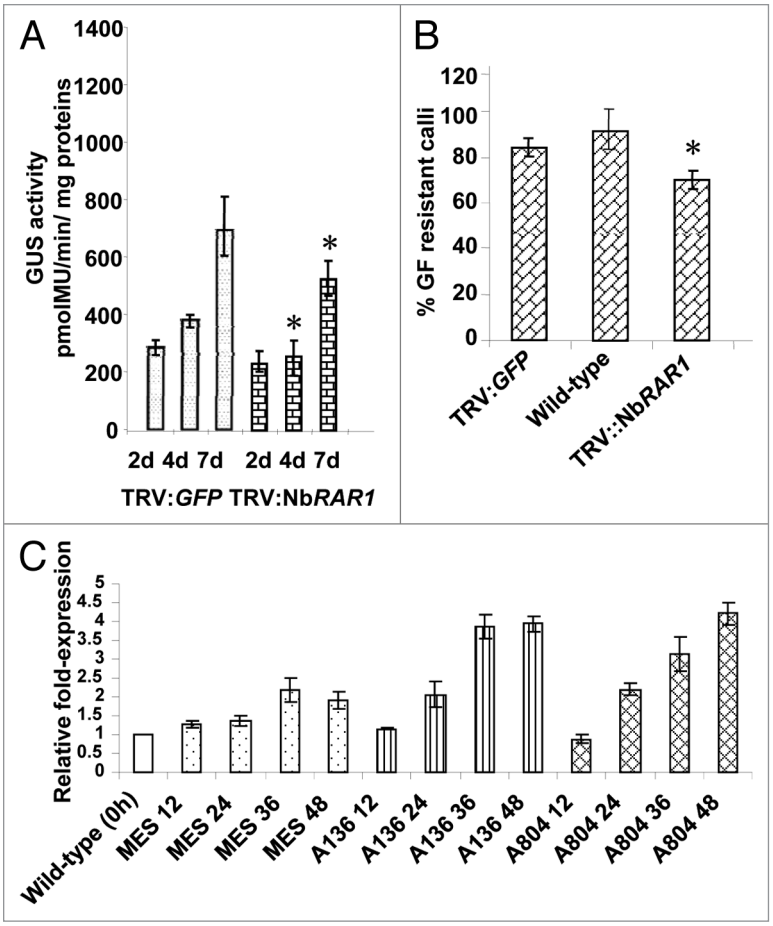

Figure 1. Transformation assays on *RAR1* gene silenced plants of *N. benthamiana* and induction of *RAR1* in response to *Agrobacterium* infection. (**A**) Transient transformation assay. Leaf disks of the gene silenced plants and control plants were inoculated with non-tumorigenic strain *A. tumefaciens* GV2260 carrying *pBISN1* (At804; has the *uidA*-intron gene within the T-DNA). Leaf disks were collected periodically and were used for measuring the fluorescence of 4-methylumbelliferone (4-MU). (**B**) Leaf disk stable transformation assay. Leaf disks of silenced and control plants were inoculated with a non-tumorigenic strain, *A. tumefaciens* GV2260, harboring the binary vector *pCAS1* (contains a *bar* gene within the T-DNA) and were incubated on callus inducing medium (CIM) with glufosinate ammonium (GF). Data represent the mean of 3 experiments with a minimum of 150 leaf disks each per treatment with their SE values shown as error bars. (**C**) Differential gene expression of *RAR1* upon *Agrobacterium* infection. Individual leaves of a minimum of 3 *N. benthamiana* plants were syringe (needleless) infiltrated with either: buffer (10 mM MES, dotted bars); an avirulent strain *A. tumefaciens* A136 (lacks Ti plasmid – cannot transfer T-DNA, line bars); or a T-DNA transfer-competent strain, *A. tumefaciens* At804 (checkered bars). Asterisks denote significant difference compared with controls using Fisher's least significant difference test at $P = 0.05$.

Table 1. qRT-PCR results showing the differential gene expression of *Arabidopsis RAR1* following *Agrobacterium* infection

Gene ID	Gene Symbol	Infected (48h)	Mock (48h)	Infected (72h)	Mock (72h)
At5g51700	*Rar1*	4.6 ± 0.5	1.9 ± 0.18	2.9 ± 0.4	1.2 ± 0.6

suggest that *RAR1* gene silencing did not affect the cell division machinery in phytohormone-rich medium. The above observations lead us to conclude that *RAR1* might be involved at the early stages of transformation.

RAR1 is induced in response to *Agrobacterium* inoculation

The expression of *RAR1* was analyzed to check whether the gene is responsive to *Agrobacterium* inoculation. To characterize *RAR1* gene expression following *Agrobacterium* inoculation, the leaves of *N. benthamiana* plants were separately infiltrated with a transfer competent strain, *A. tumefaciens* At804, a transfer deficient strain, *A. tumefaciens* A136, and the buffer control as described.[14] *RAR1* showed differential expression following both *Agrobacterium* and buffer inoculations. *RAR1* gene induction was greater (1.5 – to 2-fold) in the leaves infected with *Agrobacterium* at 24–48 h post-infection as compared with buffer inoculated plants (**Fig. 1C**). These results suggest that *RAR1* is induced upon both general stress and also upon *Agrobacterium* infection. This finding complements the work we previously published involving microarray analysis on uninfected and *Agrobacterium* infected Arabidopsis (Col-0) leaves.[15] At*RAR1* induction (~2.5-fold) was observed at 48hpi, while a relatively lower differential expression (< 1.5-fold) was observed at 72 hpi.[15] We further reconfirmed the above data through quantitative RT-PCR (qRT-PCR) analyses on *Agrobacterium* infected and uninfected leaf samples (**Table 1**) and the results were consistent with the microarray data.

Arabidopsis rar1 mutant is recalcitrant to *Agrobacterium*-mediated root transformation

Using Arabidopsis *rar1* (*Ler-rar1*)[16] mutant, the function of RAR1 protein in *Agrobacterium*-mediated plant transformation in another plant species was confirmed. **Figure 2** summarizes the root transformation assay results[15] for the mutant along with its corresponding wild-type. A significant reduction in tumor formation was observed in the *rar1* mutant as compared with the wild-type plant upon inoculation with tumorigenic strain *A. tumefaciens*, A208 (**Table 2**, **Figure 2A**). The *rar1* mutant was also evaluated for stable transformation phenotype by scoring for phosphinothricin (ppt)-resistant calli following infection with disarmed strain *Agrobacterium* At872 (T-DNA carries the *bar* gene) (**Fig. 2B**). Consistent with the tumor assay (**Fig. 2A**), we observed significantly lower stable transformation of *rar1* mutant when infected with a disarmed *Agrobacterium* strain (**Table 2**). Consistent with the Nb*RAR1* gene silenced data, the *rar1* mutant was also found to be slightly defective in transient gene expression, as seen through the reduced GUS activity following infection with *Agrobacterium* strain containing the *uidA*-intron in the T-DNA of the binary vector (**Fig. 2C** and **D**). Taken together, the data from both gene silencing in *N. benthamiana* and mutant analysis in Arabidopsis imply the role of *RAR1* in the early stages of *Agrobacterium*-mediated plant transformation.

In conclusion, we show that *RAR1* silenced *N. benthamiana* plants and *rar1* Arabidopsis mutant are partially recalcitrant to *Agrobacterium*-mediated plant transformation. The *RAR1* gene

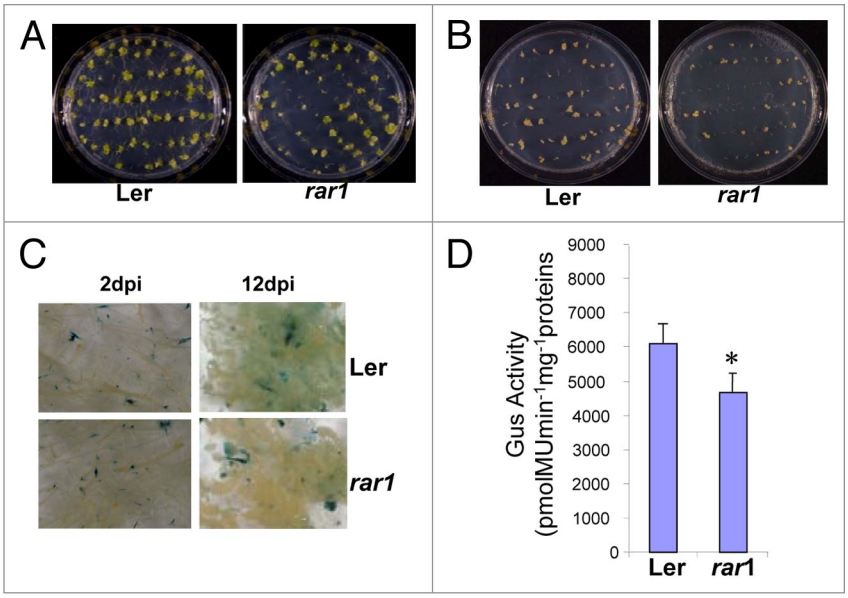

Figure 2. Root transformation assays in the Arabidopsis *rar1* mutant. (**A**) Root tumorigenesis assays. Roots of wild-type (Ler) and *rar1* mutant plants were infected with a tumorigenic strain *A. tumefaciens* A208 (nopaline strain), and tumors incited on the roots were visualized and scored 4 weeks after infection. (**B**) Stable root transformation assays. The root segments of the mutant and the wild-type (Ler) plants were inoculated with a disarmed strain, *A. tumefaciens* At872, containing the *bar* gene within the T-DNA and were incubated on callus-inducing medium (CIM) with glufosinate ammonium (GF). Photographs were taken 4 weeks after inoculation. (**C**) Roots of the wild-type and mutant plants were inoculated with a disarmed strain *A. tumefaciens* GV3101 carrying *pBISN1*. The inoculated roots were periodically collected at 2 and 12 dpi and stained with X-Gluc. (**D**) Quantification of GUS activity. The root segments were collected at 12 dpi (**C**) and were used for measuring the fluorescence of 4-methylumbelliferone (4-MU). All the experiments were repeated 2 times and obtained similar results. Asterisk denotes significant difference compared with controls using Fisher's least significant difference test at $P = 0.05$.

Table 2. Root transformation assays in *Arabidopsis* wild-type and *rar1* mutant

Root tumorigenesis assay	
Genotype	**% Tumor (total # roots) 0.D$_{600}$ (0.5)**
Ler	79.3 ± 6.2 (670) [a]
rar1	62.3 ± 9.2 (670) [b]
Callus assay (GF-resistant calli)	
Genotype	**%GF-resistant calli (total # roots) 0.D$_{600}$ (0.5)**
Ler	75.3 ± 7.7 (404)[a]
rar1	62.3 ± 7.7 (404)[b]

silenced plants and the *rar1* mutant showed reduced transient and stable transformation and is similar to the previously published data on *SGT1* silenced plants and *Arabidopsis sgt1* mutant.[2] The differential expression of the *RAR1* gene upon *Agrobacterium* infection and its known association with SGT1 might suggest a role in plant defenses against *Agrobacterium*-infection. Current research suggests that SGT1 and RAR1 associate as co-chaperones with HSP90 and with a possible role in resistance (R) protein activation.[17] Furthermore, SGT1 and RAR1 are thought to function in disease resistance by forming multiple protein complexes influencing the conformation of R protein complexes and targeting proteins for degradation.[17] The data from this manuscript and the recently published data[2] suggest that E3 ubiquitin-ligases are also involved in *Agrobacterium*-mediated plant transformation. The definite role of these genes in *Agrobacterium*-mediated plant transformation warrants further research.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This work was supported by the Samuel Roberts Noble Foundation and in part by a grant from the National Science Foundation (grant IOB 0445799)

References

1. Magori S, Citovsky V. The role of the ubiquitin-proteasome system in *Agrobacterium tumefaciens*-mediated genetic transformation of plants. Plant Physiol 2012; 160:65-71; PMID:22786890; http://dx.doi.org/10.1104/pp.112.200949
2. Anand A, Rojas CM, Tang Y, Mysore KS. Several components of SKP1/Cullin/F-box E3 ubiquitin ligase complex and associated factors play a role in *Agrobacterium*-mediated plant transformation. New Phytol 2012; 195:203-16; PMID:22486382; http://dx.doi.org/10.1111/j.1469-8137.2012.04133.x
3. Zaltsman A, Lacroix B, Gafni Y, Citovsky V. Disassembly of synthetic *Agrobacterium* T-DNA-protein complexes via the host SCF(VBF) ubiquitin-ligase complex pathway. Proc Natl Acad Sci U S A 2013; 110:169-74; PMID:23248273; http://dx.doi.org/10.1073/pnas.1210921110
4. Tzfira T, Vaidya M, Citovsky V. Involvement of targeted proteolysis in plant genetic transformation by *Agrobacterium*. Nature 2004; 431:87-92; PMID:15343337; http://dx.doi.org/10.1038/nature02857
5. Zaltsman A, Krichevsky A, Loyter A, Citovsky V. *Agrobacterium* induces expression of a host F-box protein required for tumorigenicity. Cell Host Microbe 2010; 7:197-209; PMID:20227663; http://dx.doi.org/10.1016/j.chom.2010.02.009
6. Liu Y, Burch-Smith TM, Schiff M, Feng S, Dinesh-Kumar SP. Molecular chaperone Hsp90 associates with resistance protein N and its signaling proteins SGT1 and Rar1 to modulate an innate immune response in plants. J Biol Chem 2004; 279:2101-8; PMID:14583611; http://dx.doi.org/10.1074/jbc.M310029200
7. Takahashi A, Casais C, Ichimura K, Shirasu K. HSP90 interacts with RAR1 and SGT1 and is essential for *RPS2*-mediated disease resistance in Arabidopsis. Proc Natl Acad Sci U S A 2003; 100:11777-82; PMID:14504384; http://dx.doi.org/10.1073/pnas.2033934100
8. Austin MJ, Muskett P, Kahn K, Feys BJ, Jones JDG, Parker JE. Regulatory role of SGT1 in early *R* gene-mediated plant defenses. Science 2002; 295:2077-80; PMID:11847308; http://dx.doi.org/10.1126/science.1067747
9. Azevedo C, Sadanandom A, Kitagawa K, Freialdenhoven A, Shirasu K, Schulze-Lefert P. The RAR1 interactor SGT1, an essential component of *R* gene-triggered disease resistance. Science 2002; 295:2073-6; PMID:11847307; http://dx.doi.org/10.1126/science.1067554
10. Azevedo C, Betsuyaku S, Peart J, Takahashi A, Noël L, Sadanandom A, Casais C, Parker J, Shirasu K. Role of SGT1 in resistance protein accumulation in plant immunity. EMBO J 2006; 25:2007-16; PMID:16619029; http://dx.doi.org/10.1038/sj.emboj.7601084
11. Liu F, Ni W, Griffith ME, Huang Z, Chang C, Peng W, Ma H, Xie D. The *ASK1* and *ASK2* genes are essential for Arabidopsis early development. Plant Cell 2004; 16:5-20; PMID:14688296; http://dx.doi.org/10.1105/tpc.017772
12. Shirasu K, Lahaye T, Tan M-W, Zhou F, Azevedo C, Schulze-Lefert P. A novel class of eukaryotic zinc-binding proteins is required for disease resistance signaling in barley and development in *C. elegans*. Cell 1999; 99:355-66; PMID:10571178; http://dx.doi.org/10.1016/S0092-8674(00)81522-6
13. Garfinkel DJ, Simpson RB, Ream LW, White FF, Gordon MP, Nester EW. Genetic analysis of crown gall: fine structure map of the T-DNA by site-directed mutagenesis. Cell 1981; 27:143-53; PMID:6276020; http://dx.doi.org/10.1016/0092-8674(81)90368-8
14. Anand A, Vaghchhipawala Z, Ryu CM, Kang L, Wang K, del-Pozo O, Martin GB, Mysore KS. Identification and characterization of plant genes involved in *Agrobacterium*-mediated plant transformation by virus-induced gene silencing. Mol Plant Microbe Interact 2007; 20:41-52; PMID:17249421; http://dx.doi.org/10.1094/MPMI-20-0041
15. Anand A, Krichevsky A, Schornack S, Lahaye T, Tzfira T, Tang Y, Citovsky V, Mysore KS. Arabidopsis VIRE2 INTERACTING PROTEIN2 is required for *Agrobacterium* T-DNA integration in plants. Plant Cell 2007; 19:1695-708; PMID:17496122; http://dx.doi.org/10.1105/tpc.106.042903
16. Muskett PR, Kahn K, Austin MJ, Moisan LJ, Sadanandom A, Shirasu K, Jones JDG, Parker JE. Arabidopsis RAR1 exerts rate-limiting control of *R* gene-mediated defenses against multiple pathogens. Plant Cell 2002; 14:979-92; PMID:12034891; http://dx.doi.org/10.1105/tpc.001040
17. Shirasu K, Schulze-Lefert P. Complex formation, promiscuity and multi-functionality: protein interactions in disease-resistance pathways. Trends Plant Sci 2003; 8:252-8; PMID:12818658; http://dx.doi.org/10.1016/S1360-1385(03)00104-3