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Article

Plant Pattern-Recognition Receptor FLS2 Is Directed for Degradation by the Bacterial Ubiquitin Ligase AvrPtoB

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Summary

Background: An important layer of active defense in plant immunity is the detection of pathogen-associated molecular patterns (PAMPs) mediated by cell-surface receptors. For the establishment of disease, pathogens depend on the ability to overcome PAMP perception and disable plant signaling pathways activated in response to PAMPs. Pattern recognition receptors (PRRs) are therefore prime targets for pathogen effectors. FLS2, its coreceptor BAK1, and EFR encode receptor-like kinases that play a role in immunity against bacterial pathogens.

Results: Here, we report that virulence of *Pseudomonas syringae* pv *tomato* DC3000 (PtoDC3000) in *Arabidopsis* is enhanced through the action of its effector AvrPtoB, which promotes degradation of FLS2. We show that AvrPtoB, through its N terminus, associates with FLS2 and BAK1, of which interaction with FLS2 is enhanced by flg22 activation. In vitro, AvrPtoB is active as an E3 ligase to catalyze polyubiquitination of the kinase domain of FLS2, a process confirmed in planta. Full enhancement of PtoDC3000 virulence appears to require the E3 ligase activity of AvrPtoB.

Conclusions: AvrPtoB, initially identified through its activation of hypersensitive resistance in tomato cultivars expressing the Pto kinase, is composed of at least two functional domains: the N terminus is responsible for interaction with Pto, and the C terminus carries an E3 ligase activity. Based on our findings, we propose that both domains of AvrPtoB act together to support the virulence of PtoDC3000 in *Arabidopsis* through their ability to eliminate FLS2 from the cell periphery, and probably also other PAMP sensors that are constitutively expressed or induced after pathogen challenge.

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Introduction

Several plant cell-surface receptors are known to sense foreign molecules and thereby activate the defense responses associated with innate immunity [1–5]. A prominent class of cell-surface receptors is the family of receptor-like kinases (RLKs), which includes the well-studied receptor for brassinosteroids BRI1 and its partner BAK1 (BRI1-associated kinase 1), required for plant growth and development [6, 7]. Other members like FLS2, EFR, and CERK1 from *Arabidopsis* serve as so-called pattern recognition receptors (PRRs). They are responsible for the perception of elicitor-active epitopes of bacterial flagellin (flg22), bacterial EF-Tu, and fungal chitin, respectively, which are typical pathogen-associated molecular patterns (PAMPs) conserved throughout whole classes of microbes. Interestingly, BAK1 associates with FLS2 upon stimulation with flg22 and is required for responses triggered by flg22, EF-Tu, and other PAMPs [8, 9]. It is probable that BAK1 (and possibly also its homologs in the so-called SERK family) associate not only with BRI1 and FLS2 but also with other PRRs [9, 10].

Non-self discrimination forms the first layer of active defense against potentially invading microbes. Upon PAMP-induced activation, PRRs stimulate a plethora of defense responses ranging from stomatal closure, MAP kinase activation, and changes in gene expression to callose deposition [11–13]. Host-adapted pathogens have evolved means to avoid and/or interfere with this first protective layer of PAMP-triggered immunity [14, 15]. Of particular interest are molecules that disturb signaling steps after PRR activation. These can be small molecules like coronatine produced by *P. syringae* pv *tomato* DC3000 (PtoDC3000), which is a mimic of the plant hormone jasmonic isoleucine and counteracts PAMP-triggered stomatal closure [11]. Importantly, many of the effectors delivered by pathogenic bacteria to the host cell cytoplasm via their Type-III-secretion systems (T3SS) appear to interfere with signaling in basal defense. For example, HopAI1 and HopPtoD2 produced by PtoDC3000 both negatively regulate MAP kinase cascades [16, 17]. Interference of PAMP-induced signaling upstream of MAP kinase cascades was also observed with the effectors AvrPto and AvrPtoB from PtoDC3000 [18]. Originally both effectors were identified as interactors with the tomato cytosolic kinase Pto, encoded by the *Pto* bacterial speck disease resistance gene. The Pto protein interacts with the R protein Prf and upon binding of the cognate bacterial effectors, this complex triggers a hypersensitive reaction, a type of programmed cell death (PCD) frequently associated with effector-triggered immunity in plants [19]. Pto shares structural and sequence homologies with kinase domains of RLKs, and recently FLS2 and EFR were found to associate physically with AvrPto, which inhibits their autophosphorylation activity [20]. AvrPtoB exhibits similarities to eukaryotic E3 ligases and has been shown to ubiquitinate Fen, another tomato cytosolic kinase highly related to Pto [21].

Here, we report that FLS2 and the cofactor BAK1 interact with AvrPtoB and that FLS2 is a target of AvrPtoB-mediated ubiquitination. This leads to a decrease in membrane-resident FLS2 and promotes susceptibility toward PtoDC3000 bacteria.

Results

FLS2 Is Decreased during PtoDC3000 Infection and upon AvrPtoB Expression

We studied FLS2 subcellular distribution in leaf tissues upon bacterial infection by confocal microscopy. Plasma membrane-resident FLS2 fused to the green fluorescent protein (GFP) was clearly decreased after infection by the adapted pathogen PtoDC3000 (Figure 1). To address whether the removal of FLS2 is mediated by effectors from PtoDC3000, we tested FLS2-GFP expression with the mutant strains PtoDC3000 ΔAvrPtoB and HrpS− [22]. Previously, AvrPtoB was reported to suppress flg22-induced callose deposition and to disturb flg22 responses upstream of MAP kinase activation, in addition to being an auto-active ubiquitin E3 ligase [18, 23]. HrpS− lacks the HrpS transcription factor required for expression of genes under control of the hrp-box, among which some effectors (e.g., AvrPto [24] and AvrPtoB [data not shown]) are found. No significant differences in FLS2-GFP signal at the plasma membrane compared to the untreated control were detectable in either strain. Instead, ΔAvrPtoB seems to slightly increase FLS2-GFP labeling of the plasma membrane. To further investigate the role of AvrPtoB, we generated transgenic plants containing FLS2-GFP and inducibly expressing one of the two effectors, AvrPtoB and AvrRpm1. Upon expression of AvrPtoB in planta, we observed a clear reduction of membrane-resident FLS2-GFP (Figure S1A available online). In cotreatments with flg22, we could find internalization of FLS2-GFP only to small number of endocytic vesicles, which may reflect the overall lower levels of FLS2 protein in the presence of AvrPtoB. AvrRpm1 was previously shown to suppress flg22-elicited callose deposition and to interact with RIN4, a plasma membrane-associated protein that negatively regulates basal defense [25]. Here, we observed that after expression of AvrRpm1 in planta, FLS2-GFP protein levels and induced FLS2 endocytosis were similar to control-treated leaves (Figure S1B).

To further support these cytological findings, FLS2 protein levels were assessed by immunoblotting in *Arabidopsis* lines that inducibly express AvrPtoB. Whereas the amount of FLS2 remained unchanged upon AvrPtoB expression in total protein extracts of untreated plants, the total level of FLS2 was strongly reduced upon flg22 stimulation of plants expressing AvrPtoB (Figure 2). This decrease could be inhibited by the addition of MG132, a known inhibitor of the proteasome [26], suggesting that FLS2 undergoes AvrPtoB-mediated proteasomal degradation. After analyzing membrane fractions (16,000 g pellet) that separate plasma membrane compartments from endosomal vesicles, we detected reduced FLS2 protein levels upon flg22 treatment, probably resulting from FLS2 endocytosis, as well as decreased levels of FLS2 in the presence of AvrPtoB alone. However, the reduction of membrane-resident FLS2 was much more pronounced when flg22 and AvrPtoB were both present. It is well known that activated FLS2 disappears from the plasma membrane via endocytosis [27], but our data indicate that AvrPtoB preferentially targets activated FLS2 for degradation.

The Kinase Domain of FLS2 Is a Substrate of AvrPtoB

The C-terminal domain of AvrPtoB functions as an auto-active ubiquitin E3 ligase and transfers multiple moieties of ubiquitin to the cytosolic tomato Fen kinase, but not to the Pto kinase of tomato [21, 28], whereas the N-terminal part (1–387 aa) of AvrPtoB suppresses PAMP-triggered responses [18, 29].

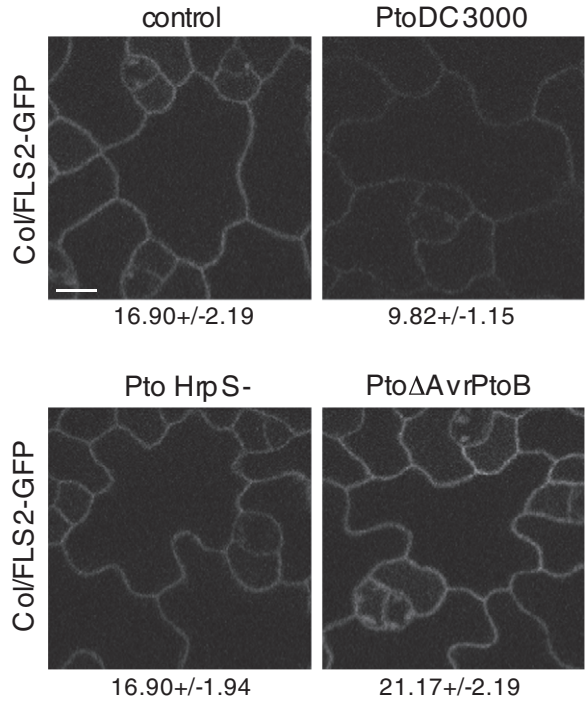

Figure 1. Plasma Membrane FLS2 Disappears by Adapted Bacterial Pathogens

Detached leaves of FLS2-GFP seedlings were incubated in a suspension without (control) or with 10^8 cfu/ml bacteria of either wild-type *Pseudomonas syringae* pv *tomato* (PtoDC3000) or the deletion strains defective in Hrp-regulated effector expression (Pto HrpS−) or devoid of AvrPtoB (PtoΔAvrPtoB). Fluorescence micrographs show optical cross-sections in epidermal cells at 5 hr after incubation. Background autofluorescence of chloroplasts is visible in stomata. For each strain, images were taken at the identical microscope settings. Representative pictures from at least three independent experiments are shown. Scale bar represents 10 μm. Numbers below are mean values and standard deviation of pixel intensity of eight areas at the plasma membrane quantified by ImageJ.

Because we observed a decrease of FLS2 in the presence of AvrPtoB, which could be inhibited by proteasome inhibitors, FLS2 ubiquitination by AvrPtoB was analyzed with in vitro ubiquitination assays. Multiple ubiquitinated forms of FLS2-CD (FLS2 cytoplasmic domain) appeared in the presence of ATP, ubiquitin, E1, Ubc9 (E2), and AvrPtoB (Figure 3A). If any of the components was left out, no ubiquitinated FLS2-CD was detected. The size difference in the laddering of ubiquitinated FLS2 relates approximately to the size of a single ubiquitin moiety. In the absence of AvrPtoB, singly ubiquitinated FLS2-CD was still detected, which could be due to ubiquitination by the E2 enzyme. This is also apparent from the analysis of total ubiquitinated proteins, where the E2 enzyme exhibited some ubiquitination activity, whereas strong auto-activity was confirmed for AvrPtoB. To confirm that the observed pattern of higher-molecular-weight forms of FLS2-CD is due to ubiquitination, an E3 ligase deletion mutant and an inactive mutant variant of AvrPtoB were analyzed. The N-terminal region (1–410 aa) lacking the E3 ligase domain as well as a point mutation in the E2 binding site (F479A) of the E3 ligase failed to generate ubiquitinated forms of FLS2-CD (Figure 3B). The auto-ubiquitination activity of AvrPtoB was also abolished. Thus, the presence of an active E3 ligase domain is required for ubiquitination of FLS2 by AvrPtoB.

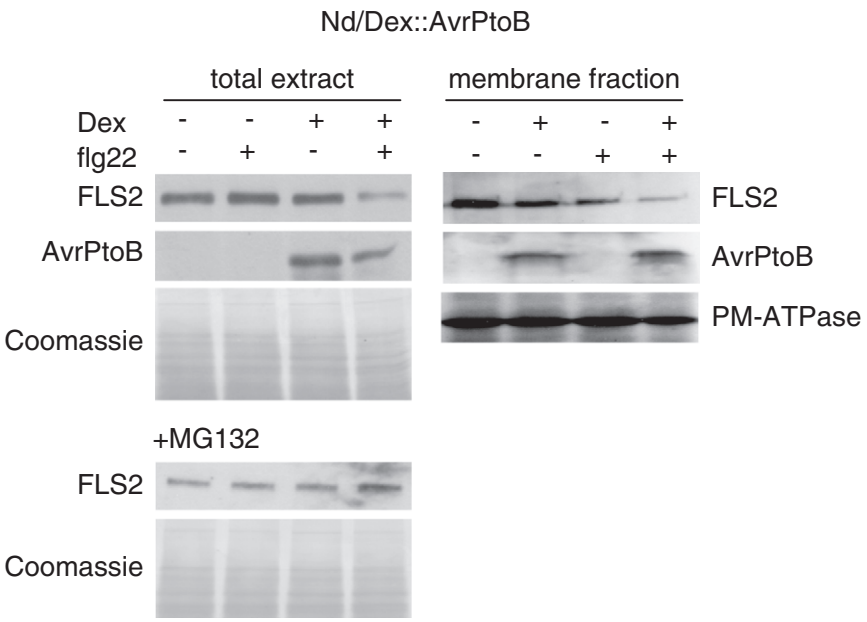

Figure 2. FLS2 Is Degraded in Response to AvrPtoB Expression In Planta

Total extracts or crude membrane preparations were obtained from Dex::AvrPtoB transgenic lines either untreated or treated with dexamethasone (2 hr), flg22 (total extract 5 min, membrane fraction 30 min), or both together, in the absence or presence of MG132. Immunoblotting was revealed with α-FLS2- and α-AvrPtoB-specific antibodies. As loading controls, Coomassie stains and protein levels of a PM-ATPase are shown. Upon expression of AvrPtoB and flg22 activation, FLS2 levels were decreased compared to controls. Similar results were obtained in at least three independent experiments.

Ubiquitin moieties are often attached to lysine residues next to a PEST-like motif [30]. FLS2 carries a well-conserved PEST-like motif in the C-terminal region of its kinase domain. Because mutational analysis demonstrated that this PEST-like motif plays a role in FLS2 endocytosis [31], we tested whether a similar mutation would affect AvrPtoB-mediated ubiquitination. Ubiquitination of the FLS2$^{\Delta PEST}$-CD variant was indistinguishable from that of wild-type FLS2-CD (Figure 3C), suggesting that AvrPtoB may not require a PEST-like motif as signal for ubiquitination.

FLS2 and EFR are both members of subgroup XII of RLKs [32], and EFR triggers a set of defense responses that overlaps with those coordinated by FLS2 [4]. It is therefore likely that AvrPtoB targets EFR in a similar manner to FLS2, and we confirmed ubiquitination of EFR-CD by AvrPtoB in vitro (Figure 4). EFR lacks a clear PEST-like motif within its cytoplasmic domain, which supports the possibility that the AvrPtoB E3 ligase activity is independent of substrate recognition via PEST-like motifs.

EFR- and FLS2-mediated signaling in response to their cognate ligands demands the function of BAK1 [8]. Because BAK1 is part of the activated FLS2 signaling complex, we tested whether AvrPtoB would also accept BAK1 as a substrate. Compared to FLS2, few ubiquitinated forms of BAK1-CD were revealed by the in vitro assay (Figure 4). We also included BRI1, the other partner of BAK1, as a possible substrate in the in vitro ubiquitination assay. Just like BAK1, few ubiquitinated forms of BRI1-CD were detected (Figure 4), which were absent when the inactive AvrPtoB (F479A) variant was provided (data not shown). To further investigate the specificity of in vitro ubiquitination, SAG101, an unrelated protein with a role in plant defense [33], was subjected to the assay. No higher forms of ubiquitinated SAG101 were observed (Figure 4), even after extended exposure (data not shown). Previously, in vitro ubiquitination of serine/threonine kinases by AvrPtoB was found to exhibit some substrate specificity [21]. In summary, our results suggest that AvrPtoB targets the cytoplasmic domains of RLKs for degradation, but FLS2 emerges as a preferred substrate in this assay.

FLS2 Is Ubiquitinated In Planta

FLS2 is ubiquitinated in vitro and degraded by AvrPtoB in planta. In order to substantiate these findings, we investigated FLS2 ubiquitination in planta. FLS2 was immunoprecipitated from lines inducibly expressing AvrPtoB, and ubiquitinated proteins were visualized with appropriate antibodies. In untreated control plants, ubiquitinated forms of FLS2 were almost undetectable (Figure 5). Upon expression of AvrPtoB, ubiquitination of FLS2 was clearly enhanced and a smear representing several ubiquitinated forms of FLS2 appeared. Interestingly, activation of FLS2 by flg22 also triggered ubiquitination of FLS2, suggesting that endogenous E3 ligases could target FLS2, possibly leading to internalization and degradation of the activated receptor. In the presence of the proteasome inhibitor MG132, FLS2 was stabilized and ubiquitinated forms of FLS2 became more visible (Figure 5). Similar results were obtained with another ubiquitin antibody, and in *fls2* mutant plants, no smear of ubiquitinated proteins was observed, demonstrating the specificity of the reaction (Figure S2). The detection of ubiquitinated forms of FLS2 in the absence of any trigger provides evidence for a constant turnover of FLS2 in unchallenged cells. However, without the stabilizing effect of MG132, ubiquitination of FLS2 seemed to be enhanced upon flg22 activation or AvrPtoB expression (Figure 5). These findings suggest that ubiquitination of FLS2 may be stimulated by ligand binding to initiate ubiquitin-mediated endocytosis and by interaction with AvrPtoB to initiate ubiquitin-mediated degradation of FLS2. It remains unclear whether these two ubiquitin-regulated processes overlap at least in some steps (e.g., through ubiquitin attachment to the same residues) or are entirely distinct from each other.

AvrPtoB Associates with FLS2

Because AvrPtoB suppresses flg22 responses upstream of the MAP kinase cascade and accepts FLS2-CD as a substrate in vitro, it is likely that AvrPtoB interacts directly with FLS2. To determine such an interaction, pull-down assays were performed. All variants (1–410 aa, F479A, and wild-type AvrPtoB) precipitated FLS2, but not the *Arabidopsis* E3 ligase At1g67800 used as control (Figure 6). This demonstrates that AvrPtoB associates with FLS2 via its N-terminal domain and the AvrPtoB E3 ligase activity is dispensable for this interaction. When the seedlings were elicited with flg22, we observed a significant increase in FLS2 precipitated

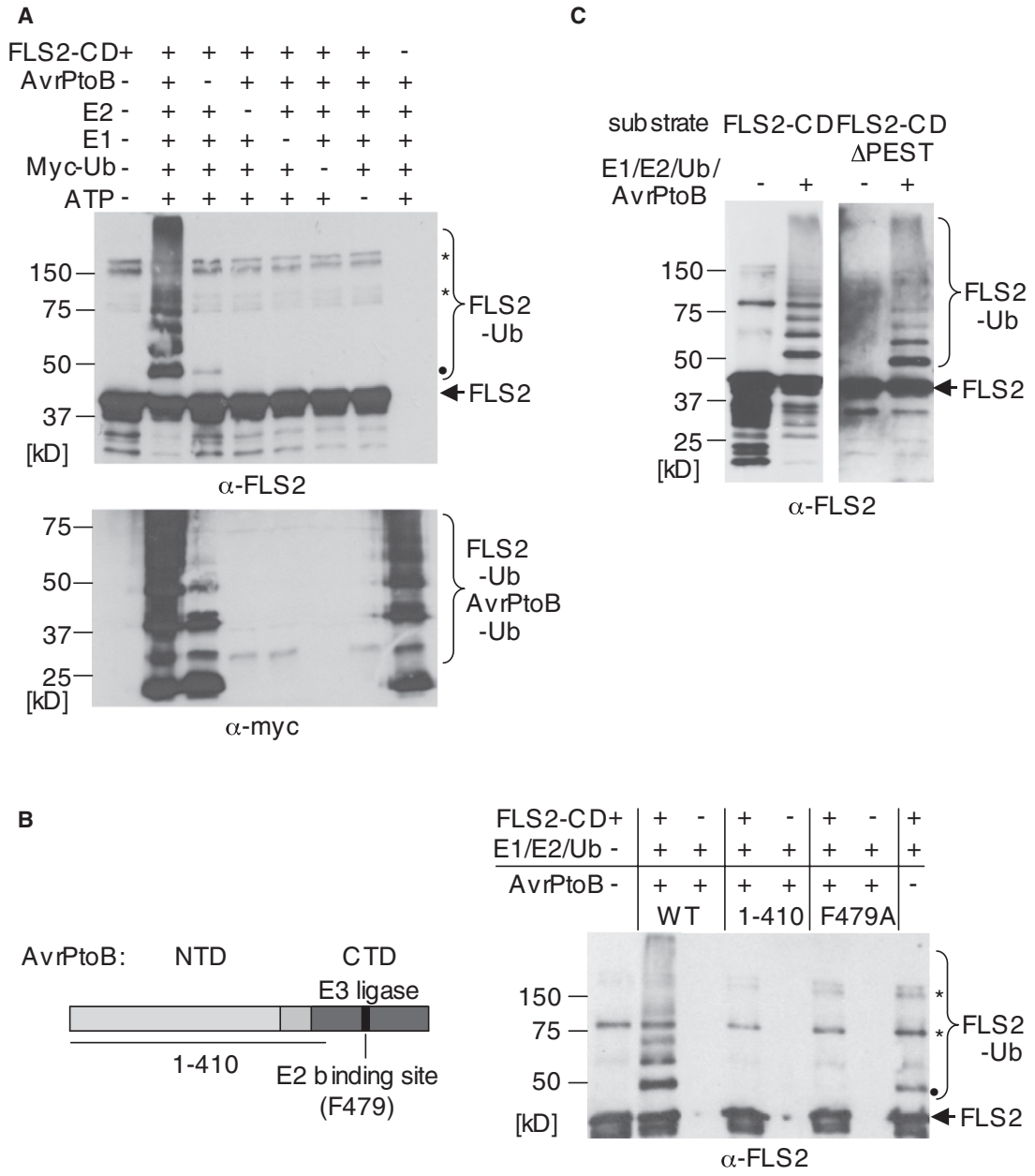

Figure 3. FLS2 Is Ubiquitinated by AvrPtoB In Vitro

(A) Purified recombinant proteins of FLS2 cytoplasmic domain (FLS2-CD-His), GST-AvrPtoB, and Ubc9-His (E2) were mixed with yeast E1, Myc-ubiquitin (Myc-Ub), and ATP as indicated. Ubiquitinated forms of FLS2 were detected with α-FLS2 antibodies, total ubiquitinated proteins were revealed by α-Myc antibodies. Ubiquitination of the FLS2 cytoplasmic domain (FLS2-Ub) and autoubiquitination of AvrPtoB (AvrPtoB-Ub) appeared as a ladder of bands separated by ~10 kDa of a single ubiquitin moiety. If any component was left out, no higher forms of ubiquitinated FLS2-CD were detected. In the absence of AvrPtoB, a single band of ubiquitinated FLS2-CD was observed (indicated by dot), which is likely due to a residual activity of the E2 enzyme. Contaminating bands because of the presence of FLS2-CD are marked by asterisk.

(B) The N-terminal region of AvrPtoB (1–410 aa) and an AvrPtoB mutant variant affecting binding of E2 (F479A) did not confer ubiquitination of FLS2-CD and were not autoactive.

(C) A mutation within the PEST-like motif of FLS2 (ΔPEST) did not impair ubiquitination of FLS2-CD by AvrPtoB. These experiments were repeated at least three times independently, with similar results.

by the N-terminal and the inactive E3 ligase variants of AvrPtoB. This was less pronounced with wild-type AvrPtoB, possibly because of its degrading activity. Together with results presented in Figure 2, this experiment provides evidence that activation of FLS2 enhances its association with AvrPtoB.

Recently, the effector AvrPto was also reported to interact with FLS2 and EFR [20]. Importantly, this interaction leads to an inhibition of the autophosphorylation activity of the FLS2 and EFR kinases. We tested whether interaction with AvrPtoB would cause a similar effect. However, even in the presence of large amounts of AvrPtoB, the 1–410 deletion fragment, or the

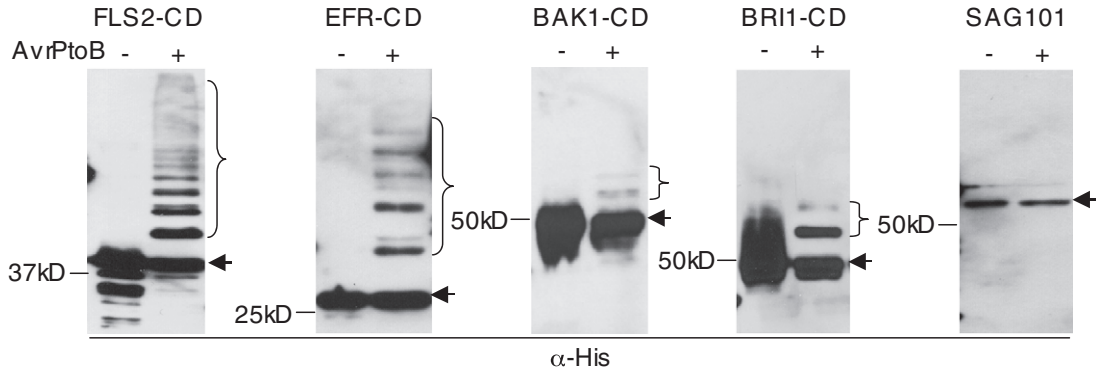

Figure 4. AvrPtoB Preferentially Ubiquitinates FLS2 In Vitro

Purified recombinant proteins of His-tagged cytoplasmic domains (CD) derived from FLS2, EFR, BAK1, BRI1, and the soluble protein SAG101 were incubated with the ubiquitination mixture with or without AvrPtoB. Immunoblotting was revealed by α-His antibodies. AvrPtoB-mediated ubiquitination of FLS2-CD and EFR-CD could be observed as a ladder of bands separated by ~10 kDa of a single ubiquitin moiety, and to a lesser extent was also detected with BAK1-CD and BRI1-CD. No ubiquitinated forms of the nonkinase protein SAG101, a component of effector-triggered resistance, could be detected. Ubiquitinated forms are indicated with brackets, nonubiquitinated substrates with arrows. Data shown are representative from three independent experiments.

F479A mutated variant, autophosphorylation activities of EFR and FLS2 remained unaltered (Figure S3). Therefore, effectors interacting with PRR kinase domains do not necessarily interfere with their kinase signaling activity. Association of AvrPtoB appeared to be independent of BAK1, because FLS2 could be precipitated from *bak1-4* mutants (Figure 6), which are strongly impaired in flg22 responses [8].

We tested additional in vitro substrates of AvrPtoB for their interaction in planta. Because we were not able to obtain transgenic lines expressing a fluorescently labeled EFR fusion protein, nor an EFR-specific antibody, we focused on BAK1 and BRI1. Although we could reveal an interaction of AvrPtoB and its variants with BAK1-GFP in untreated seedlings, we observed only slight enhancement of the interaction upon flg22 addition (Figure 6). Thus, unlike FLS2, the activation state of the BAK1 kinase and the recruitment of BAK1 into the active flg22 signaling complex seem to play a minor role in the association with AvrPtoB. Similar results were obtained (data not shown) with transgenic lines expressing a BAK1-myc fusion protein [8]. The second known partner for BAK1, BRI1, appeared not to associate with AvrPtoB. Pull-down experiments with wild-type or AvrPtoB deletion and mutant variants revealed almost no detectable BRI1-GFP signals, suggesting that BRI1 is unlikely to be a relevant target of AvrPtoB (Figure 6).

By contrast, plasma membrane-resident FLS2 and the cofactor BAK1 appear to be likely in planta targets of AvrPtoB. Because of their subcellular localization, we supposed that AvrPtoB would be associated at least partially with plasma membranes. However, cell fractionation showed that AvrPtoB

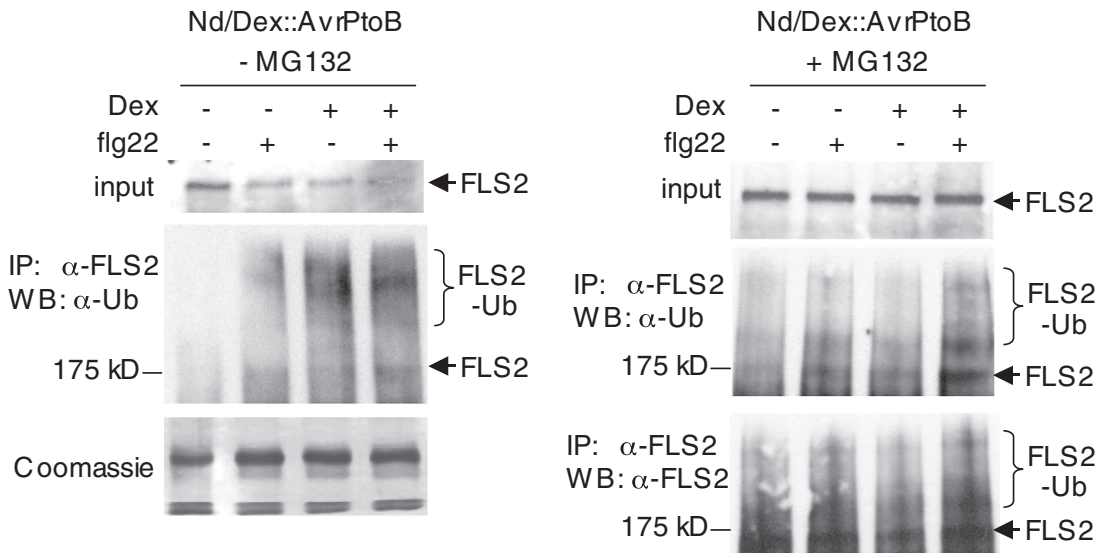

Figure 5. FLS2 Is Ubiquitinated In Vivo

Total extracts were prepared in either the presence or absence of MG132, from control or dexamethasone (2 hr) and/or flg22-treated (5 min) Dex::AvrPtoB transgenic lines. Immunoprecipitation was performed with α-FLS2 antibodies and precipitates were revealed with α-Ub (α-ubiquitin, rabbit) and α-FLS2 antibodies. Ubiquitinated forms of FLS2 (FLS2-Ub) were observed as high-molecular-weight smears. Coomassie stains of the antibody IgGs are shown as loading control for immunoprecipitation. Similar results were obtained in three independent experiments.

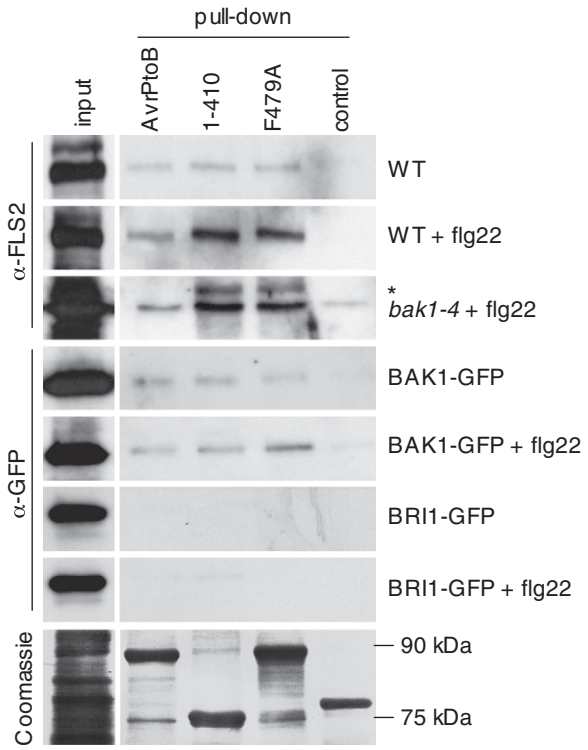

Figure 6. AvrPtoB Physically Associates with FLS2 and BAK1

Purified recombinant proteins of GST-tagged full-length AvrPtoB, its N terminus (1–410 aa), its E3 ligase inactive variant (F479A), and At1g67800 as control were bound to beads as indicated, and total seedling extracts were passed over for pull-down analysis. Immunoblotting was revealed by α-FLS2- and α-GFP-specific antibodies. AvrPtoB was able to pull down FLS2, BAK1-GFP, but not BRI1-GFP. Interaction with FLS2 was independent of the AvrPtoB E3 ligase activity and occurred through the AvrPtoB N terminus. The AvrPtoB/FLS2 association was enhanced by prior flg22 treatment (5 min) of the plant extract and did not require BAK1. A representative Coomassie stain is shown as control for equal loading of AvrPtoB, 1-410, F479A, and the control. Similar results were obtained in four independent experiments. The E3 ligase used as a control was mostly negative and only weakly precipitated FLS2 from flg22-elicited *bak1-4* mutants. The asterisk labels a band of 200 kDa that might correspond to modified forms of FLS2.

was present in the cytosol only (Figure S4A). The cytosolic localization of AvrPtoB might indicate a transient interaction with FLS2 in an intracellular compartment such as endosomes, which could also account for the increased interaction after activation of FLS2 by flg22. Additionally, some AvrPtoB was found to be associated with membranes of AvrPtoB-expressing plants in the presence of flg22 when FLS2 is ubiquitinated, as indicated by a smear above FLS2 (Figure S4B). Because our data strongly supported FLS2, and to a lesser extent BAK1, as targets of AvrPtoB, we examined the subcellular distribution of BAK1-GFP in response to bacterial infection. Unlike FLS2 (see Figure 1), we did not observe any difference in BAK1-GFP abundance after challenge with PtoDC3000 (Figure S5). This indicates that BAK1 might not be targeted for degradation by AvrPtoB in planta and stresses the role of FLS2 in AvrPtoB virulence activity.

AvrPtoB Contributes to PtoDC3000 Virulence

If AvrPtoB suppresses PAMP-triggered defense responses by targeting FLS2 for degradation, it should promote PtoDC3000 virulence. We therefore analyzed bacterial proliferation of various PtoDC3000 AvrPtoB deletion strains after leaf-surface inoculation of *Arabidopsis* wild-type plants and *fls2* mutants. In wild-type plants, the double AvrPto/AvrPtoB deletion strain was most strongly reduced in PtoDC3000 virulence, although the single AvrPtoB deletion strain showed intermediate bacterial growth rates (Figure 7). Although the PtoDC3000 single AvrPtoB deletion strain provided with the N-terminal fragment of AvrPtoB (1–387 aa) was shown to mediate Fen-triggered PCD in tomato [21], it remained compromised in its virulence activity on *Arabidopsis*. This provides evidence that the AvrPtoB E3 ligase activity plays a role in the establishment of infection by PtoDC3000 in *Arabidopsis*. In *fls2* mutants, bacterial numbers were clearly increased for all the PtoDC3000 strains tested. Despite the fact that differences in bacterial growth were only marginal, the mutant bacteria appeared still somewhat reduced in virulence compared to PtoDC3000. This suggests that additional PAMP perception systems are targets of AvrPtoB.

Discussion

Our data demonstrate that PRRs are targets of PtoDC3000 secreted effectors. In particular, we have shown that AvrPtoB inactivates PAMP signaling by clearing the FLS2 receptor from the cell membrane via ubiquitination. Two other bacterial effectors are known to directly target PRRs. DspA/E from *Erwinia amylovora* was found to interact with several RLKs [34], and AvrPto was reported to inhibit kinase activities of FLS2 and EFR by physical association [20]. Our data reveal that the interaction of AvrPtoB with FLS2 and BAK1 is independent of its enzymatic activity and occurs via its N terminus (1–410 aa). A similar region (1–387 aa) was previously identified as competent for suppression of PAMP-triggered responses [18, 29] and it largely overlaps with the domain required for interaction with tomato Pto (1–307 aa). On its own, this smaller domain is not sufficient to inhibit signaling in response to PAMPs in *Arabidopsis* cells, but it still promotes virulence in susceptible tomato plants [29, 35]. Pto encodes a soluble serine/threonine kinase, which initiates PCD via the R protein Prf [19]. Recent data point to the possibility that Pto evolved in tomato to resume the function of a decoy that traps AvrPto, and possibly also AvrPtoB, preventing them from targeting PAMP receptors [20].

Previously, AvrPtoB was demonstrated to exhibit eukaryotic ubiquitin E3 ligase activities [21, 28]. Our in vitro results show that the kinase domains of FLS2 and EFR are substrates of the AvrPtoB E3 ligase activity, whereas BAK1 and BRI1 are only weak substrates and the unrelated protein SAG101 is not. Ubiquitinated forms of FLS2 were also identified in planta in the presence of AvrPtoB alone, produced as a transgene. This suggests that AvrPtoB recruits the plant's ubiquitination machinery to exert its E3 ligase activity. Exploitation of the plant ubiquitination machinery is also known for other effectors. The PtoDC3000-derived effector HopM1 mediates proteasomal degradation of the ARF-GEF Min7 [36]. However, unlike AvrPtoB, it does not carry ubiquitination activity itself, but acts as an adaptor recruiting the plant's degradative machinery. The small molecule SylA secreted by *P. syringae* pv *syringae* was recently identified as an inhibitor of the eukaryotic proteasome [37]. Another small molecule, coronatine, which is produced by PtoDC3000, targets the plant F-box protein COI1 [11]. Molecular mimicry of eukaryotic F-box proteins is used by the *Ralstonia solanacearum* GALA effectors [38], by the *Agrobacterium* protein VirF [39], and by the viral protein P0 [40].

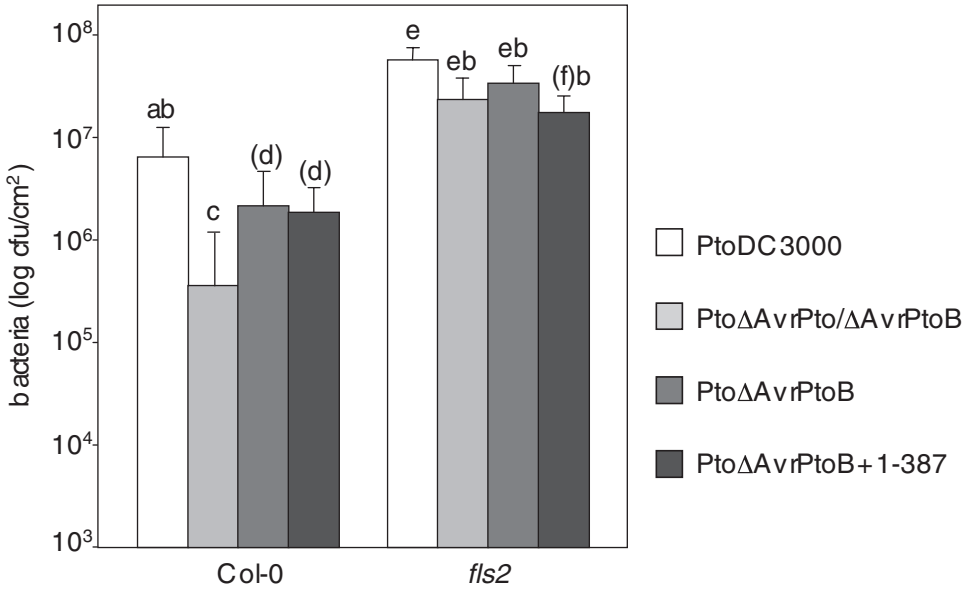

Figure 7. AvrPtoB Contributes to the Virulence Activity of PtoDC3000

Arabidopsis Col-0 wild-type and *fls2* mutant plants were infected with the indicated bacterial strains [25] by surface inoculation. In planta grown bacteria were extracted from surface-sterilized leaves at 3 days after inoculation. PtoDC3000 strains lacking AvrPtoB were compromised in virulence compared to wild-type strains but more virulent than strains devoid of both AvrPto and AvrPtoB. All strains exhibited increased virulence on *fls2* mutants. Depicted are average values and errors represent standard deviation; n = 6 plants. Letters indicate significant differences in mean log bacterial count at the level of $p < 0.05$ as revealed by multiple pairwise comparisons according to standard posthoc ANOVA analysis. Brackets indicate that multiple independent trials were necessary to reach significance.

Ubiquitination of receptor kinases is a widespread phenomenon even though it is not well studied in plants. The rice RLK Xa21 required for resistance against *Xanthomonas oryzae oryzae* interacts with the E3 ligase XB3 [41]. Mammalian EGFR (epidermal growth factor receptor), a receptor tyrosine kinase, is ubiquitinated by Cbl [42, 43]. Ligand binding leads to endocytosis and degradation of EGFR, but there is also evidence that endocytosis is independent of ubiquitination [44] or requires a deubiquitination step [45]. Furthermore, the Toll-like receptors TLR4 and TLR9, important in animal innate immunity, are ubiquitinated by Triad3A, resulting in their degradation [46].

AvrPtoB most likely targets the kinase domains of several PRRs [47], and perhaps other, as yet unknown, kinases to suppress PAMP-triggered responses. It has been postulated that acquisition of eukaryotic E3 ligase activity by AvrPtoB evolved initially to target Fen, a member of the tomato Pto kinase family, in order to circumvent Fen-triggered PCD [21], and subsequently has become important for PtoDC3000 virulence because of its ability to target PRRs. Our findings do not support the idea that the C-terminal E3 ligase domain of AvrPtoB evolved specifically to overcome Fen-triggered immunity. It is equally plausible that AvrPtoB originally acquired its E3 ligase domain to reinforce inactivation of PRRs and the tomato Fen kinase may thus be an additional target. Tomato may then have evolved Pto, a decoy kinase resistant to AvrPtoB, as proposed in previous work [21].

Proliferation of an AvrPtoB-deficient PtoDC3000 strain was clearly reduced on *Arabidopsis* wild-type plants, and to a lesser extent on *fls2* mutant lines. Previously, nonadapted *P. syringae* pv *phaseolicola* strains provided with AvrPtoB were found to exhibit increased virulence in *Arabidopsis*, and FLS2 was linked with AvrPtoB-mediated virulence [23]. Although the AvrPtoB N terminus (1–387 aa) is sufficient to interact with PRRs and to suppress PAMP-elicited responses [18, 29, 47], the AvrPtoB E3 ligase appears to be necessary for full virulence of PtoDC3000. HopPmaL is an effector encoded by *P. syringae* pv *maculicola*, which is highly related to AvrPtoB but lacks the E3 ligase domain [48]. Nevertheless, the virulence activity of HopPmaL to tomato is retained and located in the N terminus of several family members [49]. The acquisition of E3 ligase activity in AvrPtoB might enhance its efficacy in promoting disease. A single AvrPtoB molecule could cause degradation of several PRR molecules through the catalytic activity of its E3 ligase to achieve a durable inhibition of PAMP-triggered responses. In contrast, HopPmaL-mediated suppression might occur in a transient manner, targeting each PRR individually and therefore requiring much higher levels of effector to be secreted by the pathogen.

With AvrPto and AvrPtoB, PtoDC3000 produces two effectors that interact with an overlapping set of PRRs to interfere with host signaling cascades in response to PAMPs. However, the distribution of both AvrPto and AvrPtoB, and its functional homolog VirPphA, within phytopathogenic bacteria is heterogeneous. Different isolates of *P. syringae* pv *syringae* contain either both or neither effector, and *P. syringae* pv *tabaci* carries only AvrPtoB [50, 51]. In general, virulence does not seem to be fully compromised even if both effectors are absent, a fact that underlines the redundancy of effector functions.

Experimental Procedures

Confocal Microscopy

Detached leaves of FLS2-GFP transgenic seedlings were incubated with bacterial strains [21, 52] at 10^8 cfu/ml for 5 hr. GFP fluorescence was observed in a Leica TCS SP2 AOBS microscope with system software LCS

versus 2.61 after excitation at 488 nm. GFP emission and chlorophyll autofluorescence were detected at emission spectra 495–545 nm and 630-730 nm, respectively. All samples were imaged with the 63× oil immersion objective at 4× zoom. Pictures were taken in line averaging of four scans.

Protein Extraction from Plants

Nd/Dex::AvrPtoB seedlings were treated as indicated with 20 μM MG132 for 15 min followed by 30 μM dexamethasone for 2 hr and induced with 10 μM flg22 for 5 or 30 min. To extract total proteins, ground seedling material was resuspended with 50 mM Tris-HCl (pH 7.6), 2% SDS, and 0.2 mM EDTA and boiled for 5 min. Tissue debris was spun down and the supernatant was analyzed by immunoblot analysis. For crude membrane extracts, ground seedling material was mixed with 25 mM Tris-HCl (pH 8.0), 150 mM NaCl, plant protease inhibitor cocktail (Sigma). Membranes were separated by centrifugation at 16,000 × g for 20 min, resuspended in 25 mM MES (pH 6.0), 3 mM $MgCl_2$, 10 mM NaCl, and analyzed by immunoblotting.

In Vitro Ubiquitination

In vitro ubiquitination was carried out as described [53]. In brief, the assay was performed with 33 ng/μl E1 from yeast, 167 ng/μl human Myc-ubiquitin in 30 μl buffer containing 50 mM Tris-HCl (pH 7.6), 2 mM $MgCl_2$, 4 mM ATP, 1 mM DTT. Protein concentrations of all substrates, AvrPtoB and variants (1–410 aa and F479A), and Ubc9 [54] were applied to an estimated ratio of 4:2:1, incubated at 30°C for 2 hr, and analyzed by immunoblot analysis.

In Vivo Ubiquitination

Nd/Dex::AvrPtoB seedlings were treated as indicated with 20 μM MG132 for 15 min followed by 30 μM dexamethasone for 2 hr and induced with 10 μM flg22 for 5 min. Seedlings were ground in TBS (25 mM TrisHCl [pH 7.6], 140 mM NaCl, 2.5 mM KCl) containing 1% SDS, protease inhibitors, 10 μM Ubiquitin-Aldehyd, and 20 μM MG132 and boiled for 5 min. Tissue debris was spun down for 10 min at 12,000 × g. The soluble fraction was diluted 10-fold in TBS. FLS2 was immunoprecipitated by α-FLS2 bound to protein G-coupled magnetic beads. Binding was carried out overnight at 4°C. The beads were washed with TBS and eluted with TBS-1% SDS. Fractions were analyzed by immunoblot analysis with α-FLS2, α-AvrPtoB, and α-Ubiquitin (Abcam, developed in rabbit).

Pull-Down Experiments

E3 ligases (AvrPtoB, 1–410 aa, F479A, and At1g67800 [54]) were expressed heterologously in *E. coli* (Rosetta). Frozen cell pellets were resuspended in TBS containing protease inhibitor cocktail and 100 μg/ml lysozyme. Cells were ruptured by sonication and soluble fractions were separated by centrifugation, bound to GST beads, and washed with TBS. Seedlings were treated as indicated with 10 μM flg22 for 5 min and ground in TBS and protease inhibitor cocktail. The extract was solubilized with 1% NP40 for 2 hr and tissue debris was removed by centrifugation. The supernatant was passed over columns containing GST-AvrPtoB, GST-AvrPtoB-1-410, GST-AvrPtoB F479A, or GST-At1g67800 bound to GSH-sepharose beads. After washing, all proteins were eluted with TBS-1% SDS. Fractions were analyzed by immunoblot analysis.

Bacterial Infections

Bacterial infections were performed as previously described [5]. In brief, PtoDC3000 strains [29] were sprayed onto the leaf surface at 0.5 × 10^8 cfu/ml. Leaves were harvested 3 days after inoculation and surface sterilized. Two leaves of each of five plants were pooled, bacteria were extracted by grinding in $MgCl_2$, and several dilutions were plated on medium containing appropriate antibiotics. An analysis of variance was performed according to the model $X_{ijk} = \mu + S_i + B_j + SB_{ij} + \varepsilon_{ijk}$, where μ is the grand mean, S_i is the strain effect, B_j represents the effect of the genetic background, and SB_{ij} is the interaction between strains and genetic backgrounds. To identify significantly different strains, a Tukey test was applied for multiple mean comparisons. For a few pair-wise comparisons showing weak mean differences, multiple independent experiments were performed and the data were analyzed with the following model: $X_{ijk} = \mu + S_i + T_j + ST_{ij} + \varepsilon_{ijk}$, where μ is the grand mean, S_i is the effect of each of two strains, T_j indicates the trial effect, and ST_{ij} represents interactions between strains and trials.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at http://www.current-biology.com/supplemental/S0960-9822(08)01489-9.

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