

PipB2 is a substrate of the *Salmonella* pathogenicity island 1- encoded type III secretion system

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Abstract

Salmonella harbors two type III secretion systems, T3SS1 and T3SS2, encoded on the pathogenicity islands SPI1 and SPI2, respectively. Several effector proteins are secreted through these systems into the eukaryotic host cells. PipB2 is a T3SS2 effector that contributes to the modulation of kinesin-1 motor complex activity. Here, we show that PipB2 is also a substrate of T3SS1. This result was obtained infecting human epithelial HeLa cells for 2 hours and was confirmed in murine RAW264.7 macrophages, and rat NRK fibroblasts. Analysis at different time points after infection revealed that translocation of PipB2 is T3SS1-dependent in epithelial cells throughout the infection. In contrast, translocation into macrophages is T3SS1-dependent during invasion but T3SS2-dependent at later time points. The N-terminal 10 amino acid residues contain the signal necessary for translocation through both systems. These results confirm the functional overlap between these virulence-related secretion systems and suggest a new role for the effector PipB2.

Keywords

Salmonella; type III secretion; PipB2; secretion signal; mammalian cells.

Abbreviations

T3SS, type III secretion system; SPI, *Salmonella* pathogenicity island; SCV, *Salmonella* containing vacuole; SIF, *Salmonella*-induced filament; cAMP, cyclic AMP; LB, Luria-Bertani medium; Km, kanamycin; Cm, chloramphenicol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

1. Introduction

Salmonella enterica is a facultative intracellular bacterium responsible for gastroenteritis and systemic infections in many animals including humans [1]. *S. enterica* virulence depends on two distinct type three secretion systems (T3SS). T3SS1 translocates proteins, called effectors, through the plasma membrane of the host cell and is necessary for invasion. T3SS2 is induced intracellularly, injects effectors through the membrane of the *Salmonella* containing vacuole (SCV), and is essential for survival and proliferation inside host cells. Both systems, however, depend on each other for efficient functioning [2], and, although most effectors are specific substrates of one T3SS, some effectors can be secreted by both. The genes encoding the structural components, many effectors and some regulators of T3SS1 and T3SS2 are located in two *Salmonella* pathogenicity islands, SPI1 and SPI2, respectively [2].

PipB2 was described as a *Salmonella* T3SS2 effector [3] with sequence similarity to a previously identified effector of the same system, PipB [4]. PipB2 is synthesized under SPI2-inducing growth conditions and upon infection of macrophages [5], where it localizes to the SCV and to *Salmonella*-induced filaments (SIFs) 12 h post-infection. PipB2 reorganizes late endosome/lysosome compartments in mammalian cells resulting in the centrifugal extension of SIFs away from the SCV along microtubules. This activity is a consequence of its kinesin-1 binding activity [6]. Since SifA, another T3SS2 effector, down-regulates kinesin-1 recruitment, PipB2 and SifA demonstrate antagonistic activities [6]. PipB2 promotes outward movement of the SCV when myosin II activity is inhibited [7]. The characteristic positioning of SCVs to juxtannuclear regions suggests that the kinesin-inhibitory action of SifA may be dominant over the effects of PipB2 at 8 to 14 h post infection. However, at later stages

of epithelial cell infection there is an outward displacement of a significant proportion of SCVs that is dependent upon host microtubules, kinesin and PipB2, and that is involved in cell-to-cell spread of *Salmonella* during infection [8].

Here, we describe the screens that we carried out to detect T3SS effectors based on the generation of fusions with a fragment of the gene *cyaA* from *Bordetella pertussis*, called *cyaA'*, encoding the catalytic domain of a calmodulin-dependent adenylate cyclase. This enzyme catalyzes the conversion of ATP to cyclic AMP (cAMP) in the presence of calmodulin. Because calmodulin is present in eukaryotic host cells, but not in bacteria, translocation of a CyaA' fusion can be detected as an increase in the level of cAMP in cell cultures infected with *Salmonella*. As a result of these screens, we found that PipB2 can be translocated into the host cell under SPI1-inducing conditions. We show here that this effector is secreted into several mammalian cell types in a T3SS1-dependent manner at different time points after infection, and that the N-terminal 10 amino acid residues contain the signal necessary for secretion through T3SS1 and T3SS2.

2. Materials and methods

2.1 Bacterial strains, bacteriophages, strain construction, and bacterial culture

Escherichia coli and *S. enterica* serovar Typhimurium strains used in this study are described in Table 1. Transductional crosses using phage P22 HT 105/1 *int201* [9] were used for strain construction [10]. The standard culture medium for *S. enterica* and *E. coli* was Luria-Bertani (LB) broth. Solid LB contained agar 1.5% final concentration. Antibiotics were used at the following concentrations: kanamycin (Km), 50 $\mu\text{g ml}^{-1}$; chloramphenicol (Cm), 20 $\mu\text{g ml}^{-1}$; ampicillin, 100 $\mu\text{g ml}^{-1}$. For SPI1-inducing conditions, *Salmonella* strains were grown overnight at 37°C in LB-0.3M NaCl medium in static conditions. For SPI2-inducing conditions, bacteria were inoculated in minimal medium at pH 5.8 (LPM) containing 80 mM 2-(N-morpholino) ethanesulfonic acid (pH 5.8), 5 mM KCl, 7.5 mM $(\text{NH}_4)_2\text{SO}_4$, 0.5 mM K_2SO_4 , 0.1% casamino acids, 38 mM glycerol, 337.5 $\mu\text{M K}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$ (pH 7.4) and 8 $\mu\text{M MgCl}_2$, and incubated 16-24 h at 37°C with shaking.

2.2 DNA amplification with the polymerase chain reaction (PCR)

Amplification reactions were carried out as previously described [11]. PCR constructs were sequenced with an automated DNA sequencer (Stab Vida, Oeiras, Portugal) to confirm that the sequence was correct.

2.3 Construction of mutants

Disruption and replacement of 34.8 kb of SPI1, from *avrA* to *invH* genes, with a Km resistance gene (mutant ΔSPI1) and of 21.4 kb of SPI2, from *ssaU* to *ssaB*, with a Cm resistance gene (mutant ΔSPI2) were performed as previously described [12], using specific primers (Table 2). The antibiotic resistance cassette introduced by the gene-targeting procedure was eliminated by recombination using the FLP helper plasmid pCP20 [12].

2.4 Transposon mutagenesis

Mutagenesis with mini-Tn5*cyaA*' was achieved by conjugation between the donor strain *E. coli* S17-1 λ pir carrying pUT/mini-Tn5*cyaA*' [13] and the recipient strains *S. enterica* serovar Typhimurium 14028, SV5030 (14028 *slrP*::Cm^r), or SV6151 (14028 Δ SPI1 Δ SPI2 *slrP*::Cm^r). Recipient strains were incubated for 30 min at 50°C prior to mating in order to temporarily inactivate host restriction and increase the frequency of conjugation [14]. Aliquots of the donor and the recipient (500 μ l) were harvested by centrifugation, mixed on a 0.45- μ m-pore-size membrane filter placed on LB plates, and incubated at 37 °C for 4 h. After mating, the mixtures were suspended in 10 mM MgSO₄ and spread on LB agar with Km.

2.5 Plasmids

Plasmids used in this study are listed in Table 1. Plasmids expressing CyaA' fusions were derivatives of pIZ1673 [11], a modification of pSIF003-R1 [15]. This plasmids were constructed as previously described [11,15] using primers listed in Table 2.

2.6 Mammalian cell culture

HeLa cells (human epithelial; ECAC no. 93021013), RAW264.7 cells (murine macrophages; ECACC no. 91062702), and NRK-49F (normal rat kidney fibroblasts; ATCC CRL1570) were cultured in DMEM supplemented with 10% foetal calf serum. 2 mM L-glutamine, 100 U ml⁻¹ penicillin, and 100 μ g ml⁻¹ streptomycin were included in the culture media. Cells were maintained in a 5% CO₂ humidified atmosphere at 37°C.

2.7 Bacterial infection of cultured cells and protein translocation assays

HeLa, RAW 264.7 or NRK cells were plated in 24-well plates at 1.5x10⁵ cells per well and incubated 24 h at 37°C with 5% CO₂. Infections under SPI1-inducing (invasive) conditions and under non-invasive conditions were carried out as previously

described [11]. Following the infections, the translocation of CyaA' fusions into the eukaryotic cells was monitored by measuring the levels of cAMP. The infected cells were lysed and the levels of cAMP in the lysates were determined using a colorimetric direct cAMP enzyme immunoassay kit (Arbor Assays) according to the manufacturer's instructions.

2.8 Molecular characterization of mini-Tn5cyaA' insertions

A semi-random, two-step PCR protocol [16], was used to amplify genomic regions adjacent to the mini-Tn5cyaA' insertions. The first reaction was carried out on bacterial colonies with primers cya1 and stACGCC or stGATAT (see Table 2), in a final volume of 25 µl, using a thermal program with the following steps: (i) initial denaturation, 2 min at 94°C; (ii) 6 cycles of denaturation (94°C, 30 s), annealing (42°C, 30 s, -1°C each cycle), and extension (72°C, 3 min); (iii) 25 cycles of denaturation (94°C, 30 s), annealing (65°C, 30 s), and extension (72°C, 3 min); and (iv) final incubation at 72°C for 7 min, to complete extension. The second reaction was carried out with primers cya2 and st1 using 1 µl of a 5-fold dilution of the product of the first reaction. The thermal program for the second reaction was: (i) initial denaturation, 30 s at 94°C; (ii) 30 cycles of denaturation (94°C, 30 s), annealing (56°C, 30 s), and extension (72°C, 2 min); and (iii) final incubation at 72°C for 7 min, to complete extension. The final product was sequenced using primers cya2 and st1. Sequence analysis was performed with molecular biology algorithms from the National Center for Biotechnology Information at www.ncbi.nlm.nih.gov.

2.9 Western blotting and antibodies

Salmonella strains were grown overnight in LB-0.3M NaCl medium without aeration. The bacteria were then pelleted by centrifugation and resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer.

Proteins from the same numbers of bacteria were separated by gradient SDS-PAGE (Mini-PROTEAN TGX precast gels, 4-15%, BioRad) and electrophoretically transferred to nitrocellulose filters for Western blot analysis using anti-CyaA 3D1 monoclonal antibodies (1:5000; Santa Cruz Biotechnology), or anti-GroEL polyclonal antibodies (1:20000; Sigma). Goat anti-mouse HRP-conjugated antibodies (1:5000; BioRad) and goat anti-rabbit HRP-conjugated antibodies (1:10000; GE Healthcare) were used as secondary antibodies.

3. Results and discussion

3.1 *CyaA*-based screens to detect T3SS effectors in *Salmonella*

Mutagenesis of *S. enterica* serovar Typhimurium strain 14028 with mini-Tn5*cyaA* was carried out in order to obtain *cyaA* translational fusions. Pools of 100 different mutants were used to infect HeLa cells under SPI1-inducing conditions, and RAW264.7 macrophages under non-invasive conditions. Following infections, the concentration of cAMP in the cell cultures was determined. Bacteria from pools that caused at least a 10-fold increase in cAMP above the background level were tested again in smaller pools and individual positive clones were finally identified. Three positive clones, termed A27.1, B4.10, and C8.4, were detected from 67 pools, representing 6700 mutants. PCR was used to amplify genomic regions adjacent to the mini-Tn5*cyaA* insertions and DNA sequences upstream *cyaA* were determined. The genes identified were *sipA* and *slrP* (twice). The products of these genes are T3SS effectors of *S. enterica*, confirming the usefulness of the analysis of CyaA fusions to detect substrates of these virulence-related secretion systems. Additional mini-Tn5*cyaA* insertions were generated in a Δ *slrP* background, to avoid the detection of the most frequently isolated gene in this kind of screens, and in a Δ *slrP*::Cm Δ SPI1 Δ SPI2 strain, since these islands contain many effector-encoding genes. Mutants obtained in this last background were pooled, lysed with bacteriophage P22, and the insertions were transferred by transduction to a wild-type background (since the islands are necessary for T3SS-dependent translocation). 22500 additional mutants were screened as above. The results of all the screens are summarized in Table 1. The genes detected encode known T3SS effectors and all of them were identified under SPI1-inducing conditions, when T3SS1 is active but T3SS2 is not expressed.

One conclusion from these results is that the CyaA'-based screens are very efficient to detect T3SS1 effectors, but conditions should be optimized in order to detect T3SS2 effectors. In addition, at least 10-fold more pools need to be tested to saturate the screen and find new effectors. Two of the effectors detected in our screens, SipA [17] and SopE2 [18], were described as T3SS1 effectors, whereas SlrP [19] and SteB [20] can be secreted through both, T3SS1 and T3SS2. The identification of PipB2 in screens carried out in HeLa cells under SPI1-inducing conditions was surprising since this effector has only been described as a T3SS2 effector. Therefore, we decided to explore this result further.

3.2 PipB2 is translocated into host cells through T3SS1

To investigate if PipB2 can be translocated through T3SS1, translocation of the PipB2-CyaA' chromosomal fusion obtained in the previous section was tested in three different genetic backgrounds: wild-type, Δ SPI1 (lacking T3SS1), and Δ SPI2 (lacking T3SS2). Bacteria were grown under SPI1-inducing conditions and cAMP was measured 2 h post-infection. As seen in Fig. 1, PipB2 was translocated into the host cells from the wild-type and the Δ SPI2 strains, but not from the Δ SPI1 strain, suggesting that T3SS1, but not T3SS2, is essential for the translocation of PipB2 under these conditions. Interestingly, similar results were obtained in three different host cell types from three different mammalian species: human epithelial HeLa cells, murine RAW264.7 macrophages, and rat NRK fibroblasts.

Figure 1

3.3 Kinetics of T3SS1- and T3SS2-dependent translocation of PipB2 into host cells

The kinetics of the translocation of PipB2 was studied in HeLa and RAW264.7 cells to analyze the contribution of T3SS1 and T3SS2. Non-phagocytic HeLa cells were infected with SPI1 induced, invasive *Salmonella*, and the levels of cAMP, indicative of translocation, were measured at five time points after infection. As seen in Fig. 2A, translocation was observed from 2 h post-infection until the end of the experiment and was T3SS1-dependent and T3SS2-independent. These results suggest that, in epithelial cells, PipB2 can be translocated through T3SS1, not only during invasion but also from inside the SCV. This is consistent with a previous report showing that the invasion-associated T3SS1 is expressed inside epithelial cells at late stages of the infection [21]. Phagocytic cells can be infected with invasive and with non-invasive bacteria. RAW264.7 were infected with invasive bacteria for the 30 min and 2 h time points, and with non-invasive bacteria for longer infections, to prevent rapid cell death induced by invasive bacteria in macrophages [22]. The results shown in Fig. 2B suggest that PipB2 is secreted through T3SS1 during invasion of macrophages (30 min and 2 h post-infection), and through T3SS2 when bacteria have established their intracellular niche (8 h and 16 h post-infection). The lack of translocation at 4 h post-infection suggests that the non-invasive bacteria used for this infection do not express any T3SS at this time point.

Figure 2

3.4 Synthesis of PipB2 under SPI1- and SPI2-inducing conditions

Previous data indicating that *pipB2* is expressed under SPI2-inducing growth conditions [3] are consistent with translocation of PipB2 through T3SS2. Our results showing that this protein is also a T3SS1 substrate suggest that *pipB2* should also be

expressed under SPI1-inducing growth conditions. To test this prediction, we took advantage of the chromosomal *pipB2-cyaA*' fusion, which is expressed from the *pipB2* promoter. Bacteria carrying the fusion were grown overnight in LPM medium (SPI2-inducing growth conditions) and in LB-0.3M NaCl without aeration (SPI1-inducing growth conditions) and the level of PipB2-CyaA' protein was measured by immunoblot with an anti-CyaA antibody. The level of PipB2 was similar in both media (Fig. 3A). This result confirms that *Salmonella* accumulates a significant amount of PipB2 when the T3SS1 is mounted.

Figure 3

3.5 Analysis of the signal sequence necessary for translocation of PipB2 through T3SS1

We generated several CyaA' fusions, containing different fragments of PipB2, that were expressed from a constitutive promoter in a plasmid. Expression was confirmed by immunoblot (Fig. 3B). Translocation of the fusions into HeLa cells (Fig. 4A) and RAW264.7 macrophages (Fig. 4B) was analyzed 16 h post-infection. In addition to the full length protein (1-350) and the 1-225 fragment, previously described as sufficient for translocation [3], the N-terminal 10 residues of PipB2 were able to direct translocation of CyaA' into both cell types. Consistent with this result, the fragment of PipB2 including amino acids 11-350 was not translocated. The N-terminal fragments 1-48 and 1-100 failed to translocate, maybe because the N-terminal secretion signal is buried due to inappropriate folding. Finally, translocation of the N-terminal fragments (amino acids 1-10 and 1-225) was examined 16 h post-infection in three different genetic backgrounds: wild-type, Δ SPI1, and Δ SPI2. Both fragments were translocated into HeLa cells in a T3SS1-dependent manner (Fig. 4C), and into

RAW264.7 cells in a T3SS2-dependent manner (Fig. 4D). These results confirm that PipB2 is a substrate of T3SS1 and T3SS2 and locate the minimal signal necessary for translocation through both systems in the N-terminal 10 amino acid residues.

Figure 4

This work has identified PipB2, a *Salmonella* effector originally described as a T3SS2 substrate, as a T3SS1 effector. This is an interesting addition to the increasing number of examples of functional overlap between both secretion systems during pathogenesis. Our work also opens the way for the exploration of additional roles of PipB2 in the interaction of *Salmonella* with host cells.

Acknowledgements

This work was supported by grant SAF2010-15015 from the Spanish Ministry of Science and Innovation and the European Regional Development Fund, and grant P08-CVI-03487 from the Consejería de Economía, Innovación y Ciencia, Junta de Andalucía, Spain. FBO is a recipient of a FPI fellowship from the Spanish Ministry of Science and Innovation, and ECM is a recipient of a predoctoral fellowship from Consejería de Economía, Innovación y Ciencia, Junta de Andalucía, Spain. We are grateful to I. Rosenshine (The Hebrew University, Israel) for kindly providing plasmids pSIF003-R1 and pUTmini-Tn5 $cyaA'$, and to E. Krin and A. Danchin (Institut Pasteur, France) for the generous gift of *E. coli* TP610.

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Figure legends

Fig. 1. T3SS1-dependent translocation of PipB2 in HeLa, RAW264.7, and NRK cells.

HeLa and NRK cells were infected at a MOI of 100:1 with bacteria that had been grown overnight without aeration in LB-0.3M NaCl. RAW264.7 cells were infected at a MOI of 100:1 with bacteria grown for 24 h with shaking in LB. *S. enterica* serovar Typhimurium used were 14028 (wild-type, wt) and derivatives lacking SPI1 or SPI2. The indicated strains carried an insertion of mini-Tn5*cyaA*' in *pipB2* that generated a translational fusion (PipB2-CyaA'). The concentrations of cAMP in the infected cultures were measured 2 h post-infection as an indication of the translocation of the PipB2-CyaA' fusion. Means and standard deviations from duplicate experiments are represented.

Fig. 2. Kinetics of translocation of PipB2 into host cells. Epithelial HeLa cells (A) or RAW264.7 macrophages (B) were infected with *S. enterica* serovar Typhimurium wild-type (wt), Δ SPI1 or Δ SPI2 strains, expressing a PipB2-CyaA' fusion generated by insertion of mini-Tn5*cyaA*' in *pipB2*. To analyze translocation, the level of cAMP was measured 30 min, 2 h, 4 h, 8 h, and 16 h post-infection. Means and standard deviations from duplicate experiments are represented.

Fig. 3. Synthesis of PipB2-CyaA' fusions. (A) Expression of PipB2 in different culture media. Western blot with anti-CyaA monoclonal antibodies was used to monitor the expression of a chromosomal PipB2-CyaA' fusion generated by a mini-Tn5*cyaA*' insertion. Bacteria were grown under SPI2-inducing conditions (LPM) or under SPI1-inducing conditions (LB). Anti-GroEL antibodies were used as loading control. (B) Expression of PipB2-CyaA' plasmid fusions. Derivatives of *S. enterica* serovar Typhimurium strain 14028 carrying plasmids expressing full-length (1-350) or different

fragments of PipB2 in fusion with CyaA' from *B. pertussis* (numbers indicate aminoacids of PipB2 included) were grown overnight in LB. The presence of the CyaA' fusions in cell lysates (from 10^8 cells) was analyzed by immunoblotting with anti-CyaA antibodies (upper panel). Anti-GroEL antibodies were used as loading control. Molecular mass markers, in kDa, are indicated on the left of each panel.

Fig. 4. Analysis of the translocation of fragments of PipB2. HeLa cells (A) or RAW264.7 cells (B) grown in 24-well plates were infected with the wild-type strain of *S. enterica* serovar Typhimurium expressing fragments of PipB2 (numbers indicate aminoacids) in fusion with the catalytic domain of CyaA from *B. pertussis*. HeLa cells (C) or RAW264.7 cells (D) were infected with *S. enterica* serovar Typhimurium wild-type (wt), Δ SPI1 or Δ SPI2 strains, expressing PipB2(1-10)-CyaA' or PipB2(1-225)-CyaA'. Concentration of cAMP was measured 16 h post-infection. Means and standard deviations from duplicate experiments are represented.

Table legends

Table 1. Bacterial strains and plasmids used in this study

Table 2. Oligonucleotides used in this study

Table 3. List of genes for T3SS effectors detected in screens with mini-Tn5*cyoA*'

Table 1

Bacterial strains and plasmids used in this study

Strain/Plasmid	Relevant characteristics	Source/Reference
<i>E. coli</i>		
DH5 α	<i>supE44</i> Δ <i>lacU169</i> (Φ 80 <i>lacZ</i> Δ M15) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	[23]
S17-1 λ pir	<i>recA</i> <i>pro</i> <i>hsdR</i> <i>RP4-2-Tc::Mu-Km::Tn7</i> λ pir	[24]
TP610	<i>F-</i> , <i>thi-1</i> <i>thr-1</i> <i>leuB6</i> <i>lacY1</i> <i>tonA21</i> <i>supE44</i> <i>hsdR</i> <i>hsdM</i> <i>recBC</i> <i>lop-11</i> <i>lig</i> ⁺ <i>cya-610</i>	[25]
<i>S. enterica</i> serovar Typhimurium ^a		
14028	Wild type	ATCC
SV5030	14028 Δ <i>slrP</i> ::Cm ^r	Laboratory stock
SV6017	14028 Δ SPI2::Cm ^r	This study
SV6055	14028 Δ SPI1::Km ^r	This study
SV6151	14028 Δ SPI1 Δ SPI2 Δ <i>slrP</i> ::Cm	This study
SV6619	14028 Δ <i>slrP</i> ::Cm ^r <i>pipB2</i> ::mini-Tn5 <i>cyaA</i> '	This study
<i>Plasmids</i>		
pKD3	<i>bla</i> FRT <i>cat</i> FRT PS1 PS2 oriR6K	[12]
pKD4	<i>bla</i> FRT <i>aph</i> FRT PS1 PS2 oriR6K	[12]
pKD46	<i>bla</i> P _{BAD} <i>gam</i> <i>bet</i> <i>exo</i> pSC101 oriTS	[12]
pCP20	<i>bla</i> <i>cat</i> <i>cI857</i> λ P _R <i>flp</i> pSC101 oriTS	[26]
pIZ1673	pSIF003-R1 Δ <i>lacI</i>	[11]
pIZ1907	pIZ1673-PipB2(1-350)	This study
pIZ1908	pIZ1673-PipB2(11-350)	This study
pIZ1911	pIZ1673-PipB2(1-10)	This study
pIZ1913	pIZ1673-PipB2(1-48)	This study
pIZ1947	pIZ1673-PipB2(1-100)	This study
pIZ1948	pIZ1673-PipB2(1-225)	This study
pUTmini-Tn5 <i>cyaA</i> '	Suicide delivery plasmid for mini-Tn5 <i>cyaA</i> '	[13]

^a Derivatives of these strains were used as indicated in the text

Table 2

Oligonucleotides used in this study

Oligonucleotide / use	Sequence 5'-3' (restriction sites are underlined)
<i>Construction of SPI1 mutant</i>	
SPI1P1	AGCATAACGGCATTGTTATCGAATCGCTCATAAAGC GTTTGTGTAGGCTGGAGCTGCTTC
SPI1P2	TATAAGGCTTGCAGTCTTTCATGGGCAGCAAGTAAC GTCTCATATGAATATCCTCCTTAG
<i>Construction of SPI2 mutant</i>	
SPI2P1	ATGTCTGAGGAGGGATTCATGCTGGCAGTTTTAAAA GGCAGTGTAGGCTGGAGCTGCTTC
SPI2P2	GGTGTTCGGTAGAATGCGCATAATCTATCTTCATCA CCACATATGAATATCCTCCTTAG
<i>Construction of pIZ1907</i>	
pipb2bampsif5'	GAATGGATCCAGGAGGACAGCTATGGAGCGTTCCT CGATAG
pipb2bampsif3'	GAATGGATCCAAATATTTTCACTATAAAAATTC
<i>Construction of pIZ1908</i>	
pipb2_11bampsif5'	GAATGGATCCAGGAGGAAATATATGGCTAAATCTGC TTTTGG
pipb2bampsif3'	as above
<i>Construction of pIZ1911</i>	
pipB2bampsif5'	as above
pipB2_10bampsif3'	CAAGGGATCCAACCAGCCAGACTATCGAGTGAAC
<i>Construction of pIZ1913</i>	
pipB2bampsif5'	as above
pipB2_48bampsif3'	CACAGGATCCACCGACGTATCCCACCACAGGTAAAA AAG
<i>Construction of pIZ1947</i>	
pipB2bampsif5'	as above
pipB2_100bampsif3'	GAATGGATCCATTCGTTATTCTCACCAGGAAG
<i>Construction of pIZ1948</i>	
pipB2bampsif5'	as above
pipB2_225bampsif3'	GAATGGATCCAATCGAGGGTAGCGCCACACATAATG
<i>ST-PCR</i>	
cya1	GAATGGGGGTTGACCAGGCGGAACATCAATGTGGC
cya2	CGGCGTTTGCCTAACCAGCC
st1	GGCCACGCGTCGACTAGTAC
stACGCC	GGCCACGCGTCGACTAGTACNNNNNNNNNNACGCC
stGATAT	GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT

Table 3List of genes for T3SS effectors detected in screens with mini-Tn5*cyaA'*

Gene	Candidate	Chormosomal location	Protein size	Amino acids in the fusion
<i>pipB2</i>	S59A3.2	Out of SPI1 and SPI2	350	1-336
<i>sipA</i>	A27.1	SPI1	685	1-610
<i>sipA</i>	S4C10.6	SPI1	685	1-519
<i>sipA</i>	S41B2.10	SPI1	685	1-557
<i>slrP</i>	B4.10	Out of SPI1 and SPI2	765	1-126
<i>slrP</i>	C8.4	Out of SPI1 and SPI2	765	1-133
<i>sopE2</i>	S37A8.5	Bacteriophage	240	1-75
<i>steB</i>	S47A2.3	Out of SPI1 and SPI2	133	1-72
<i>steB</i>	T122A2.7	Out of SPI1 and SPI2	133	1-72

Figure 1
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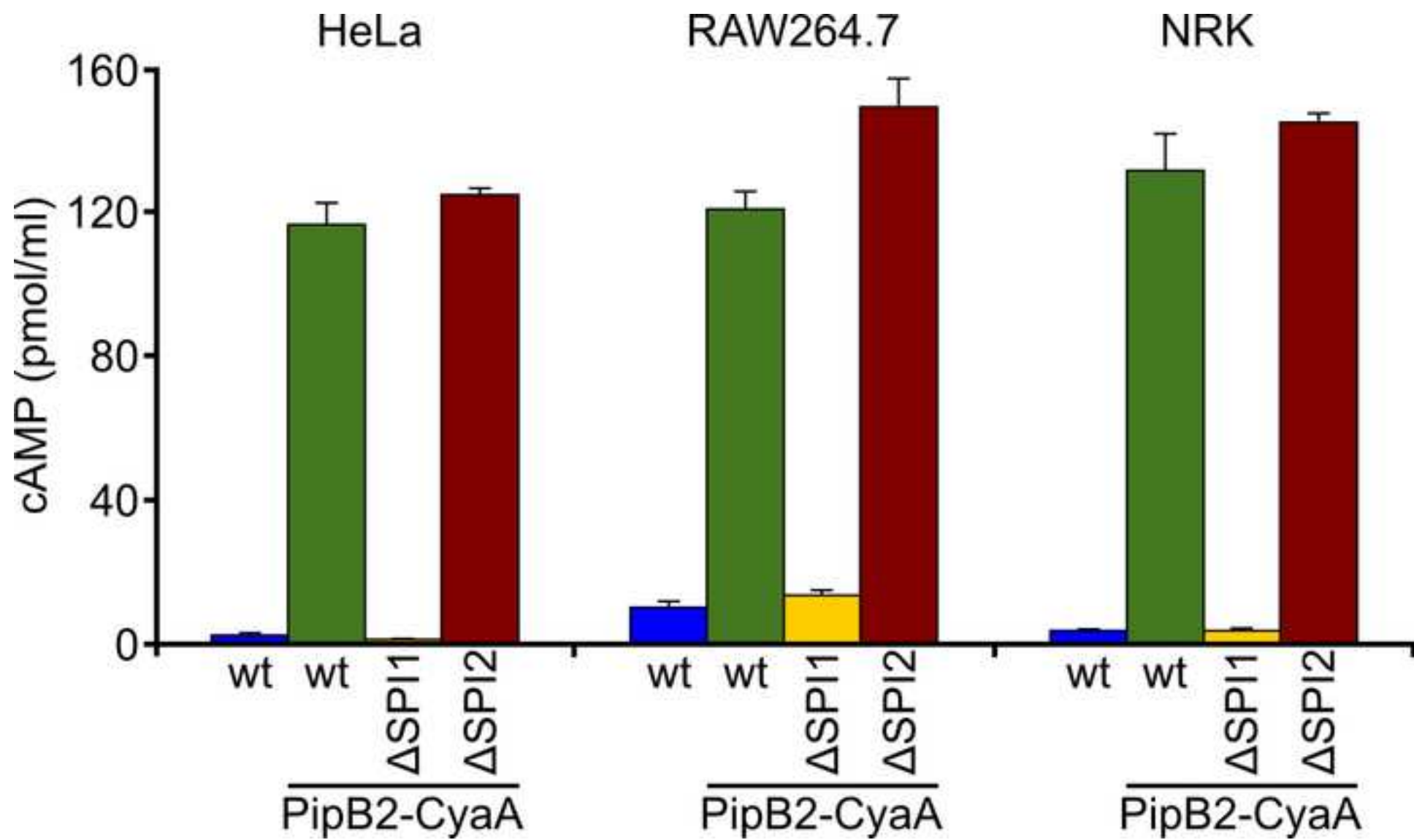


Figure 2
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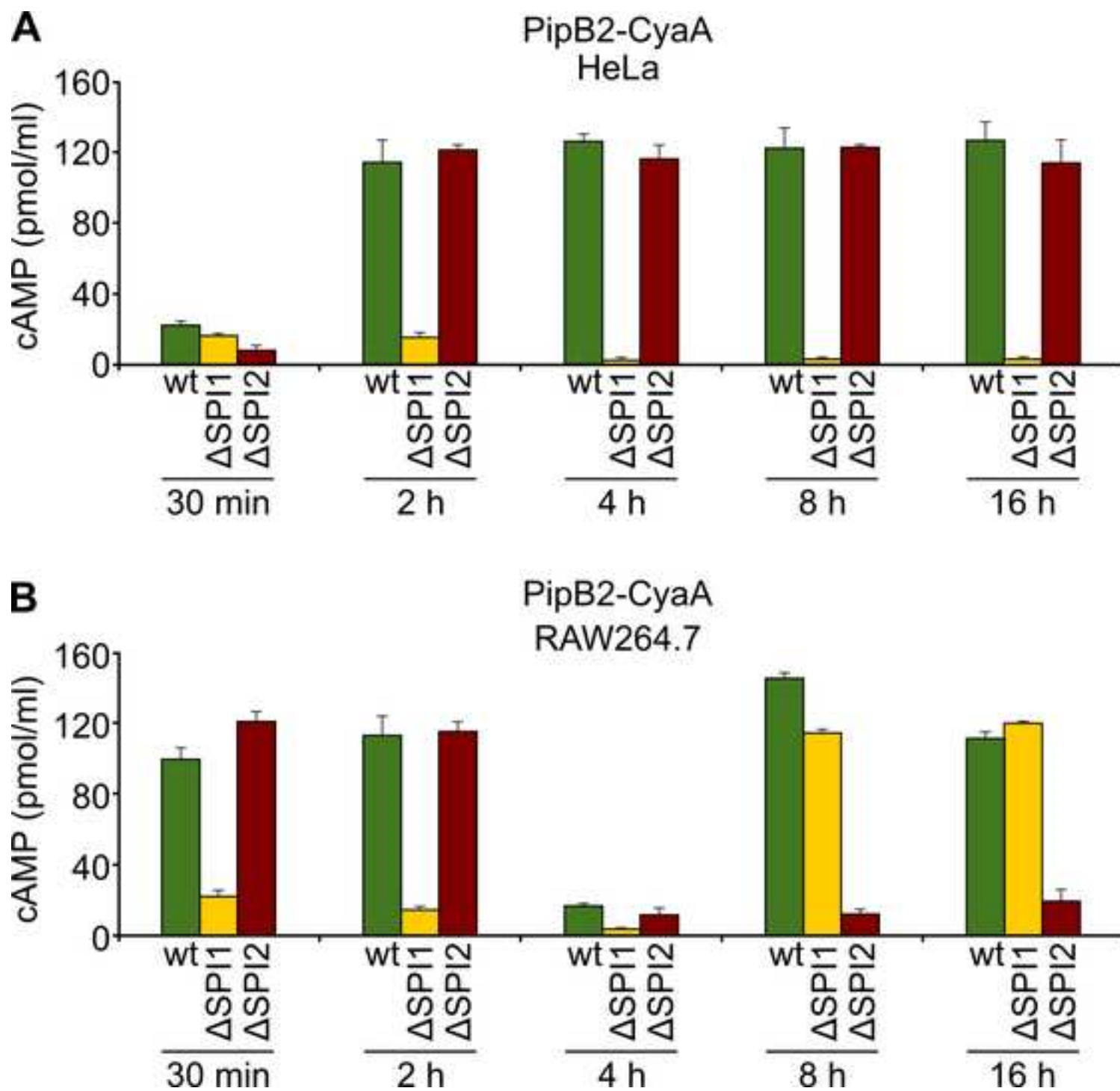


Figure 3
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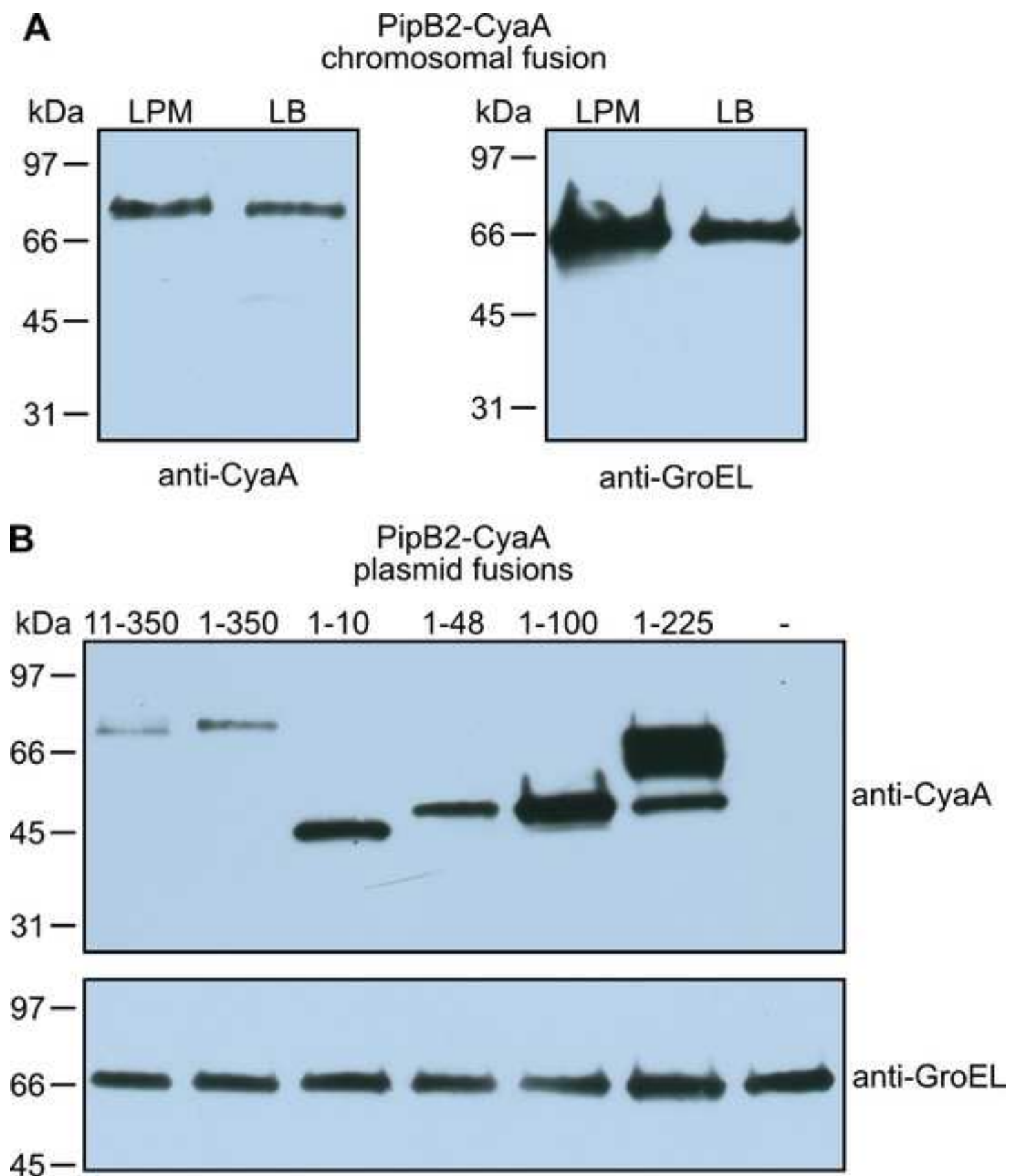


Figure 4
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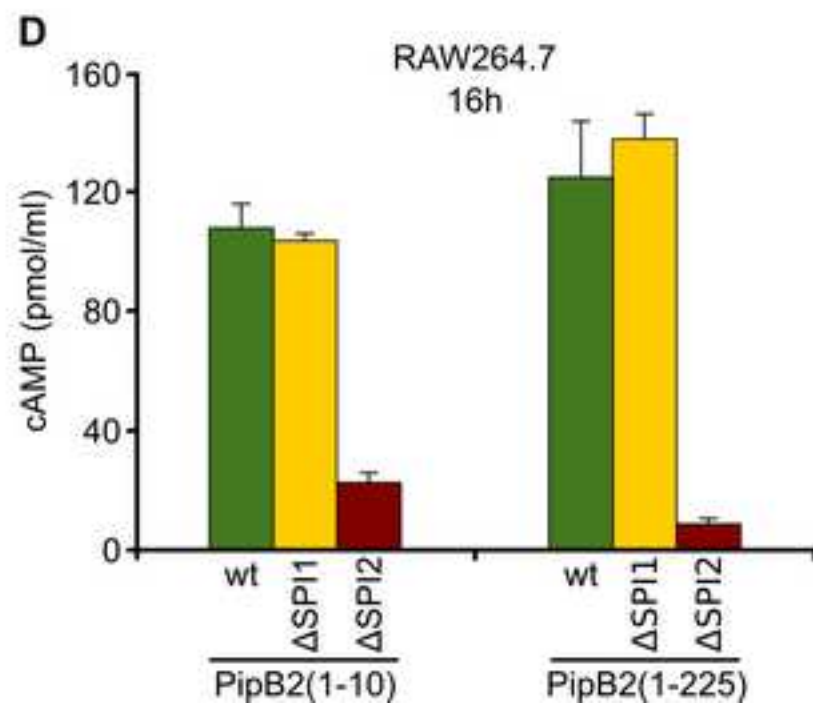
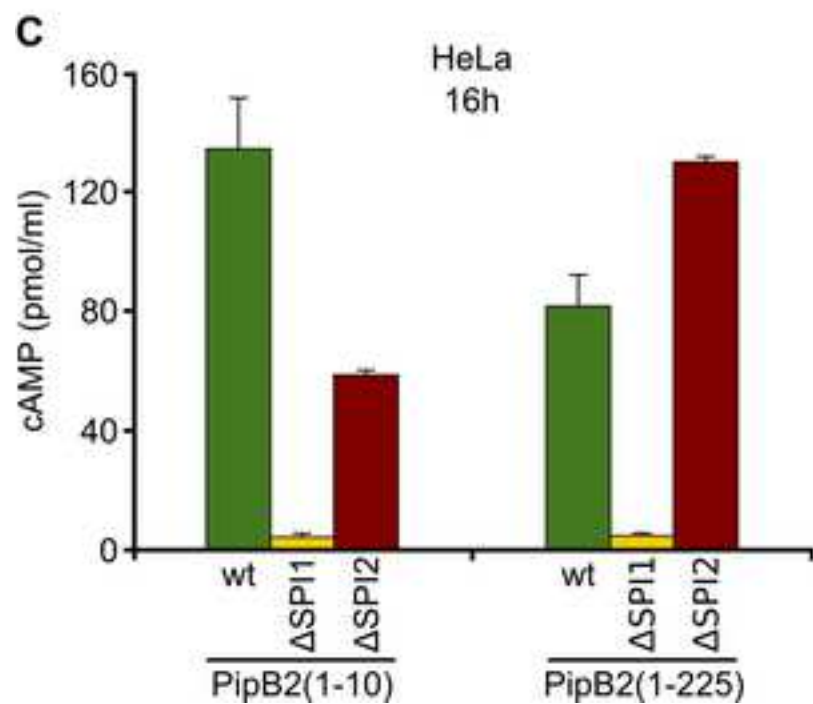
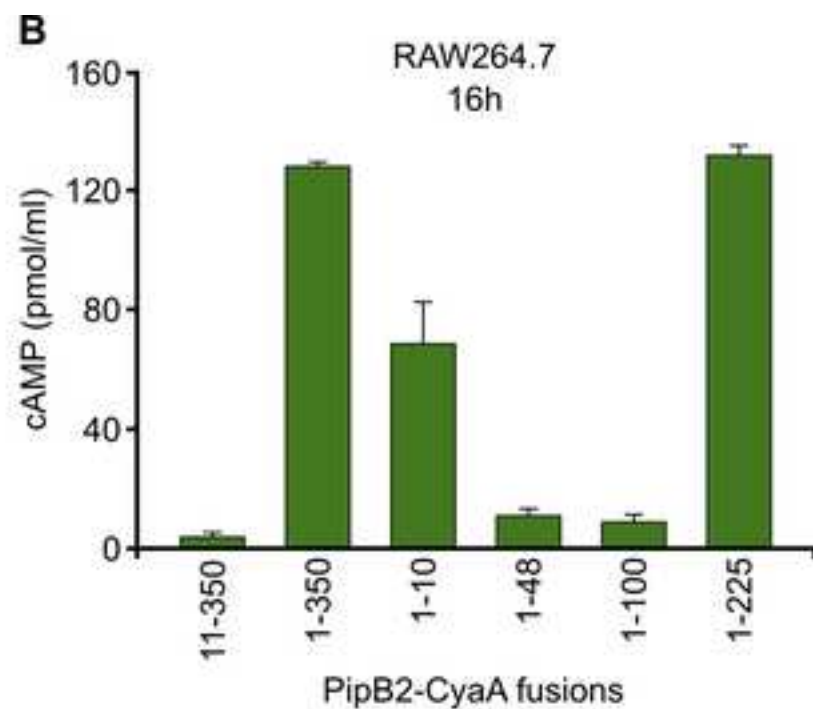
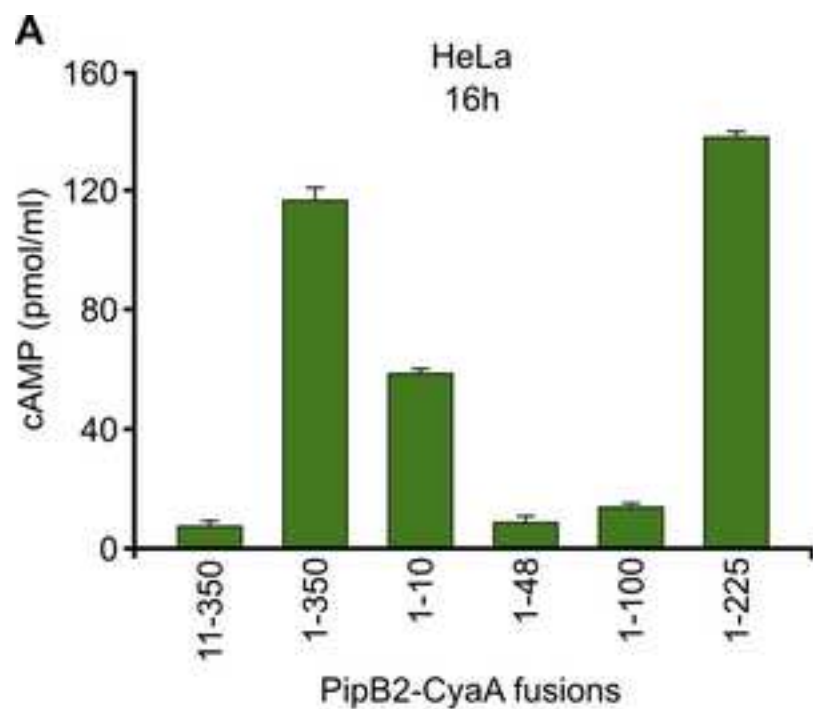


Figure 1 b/w
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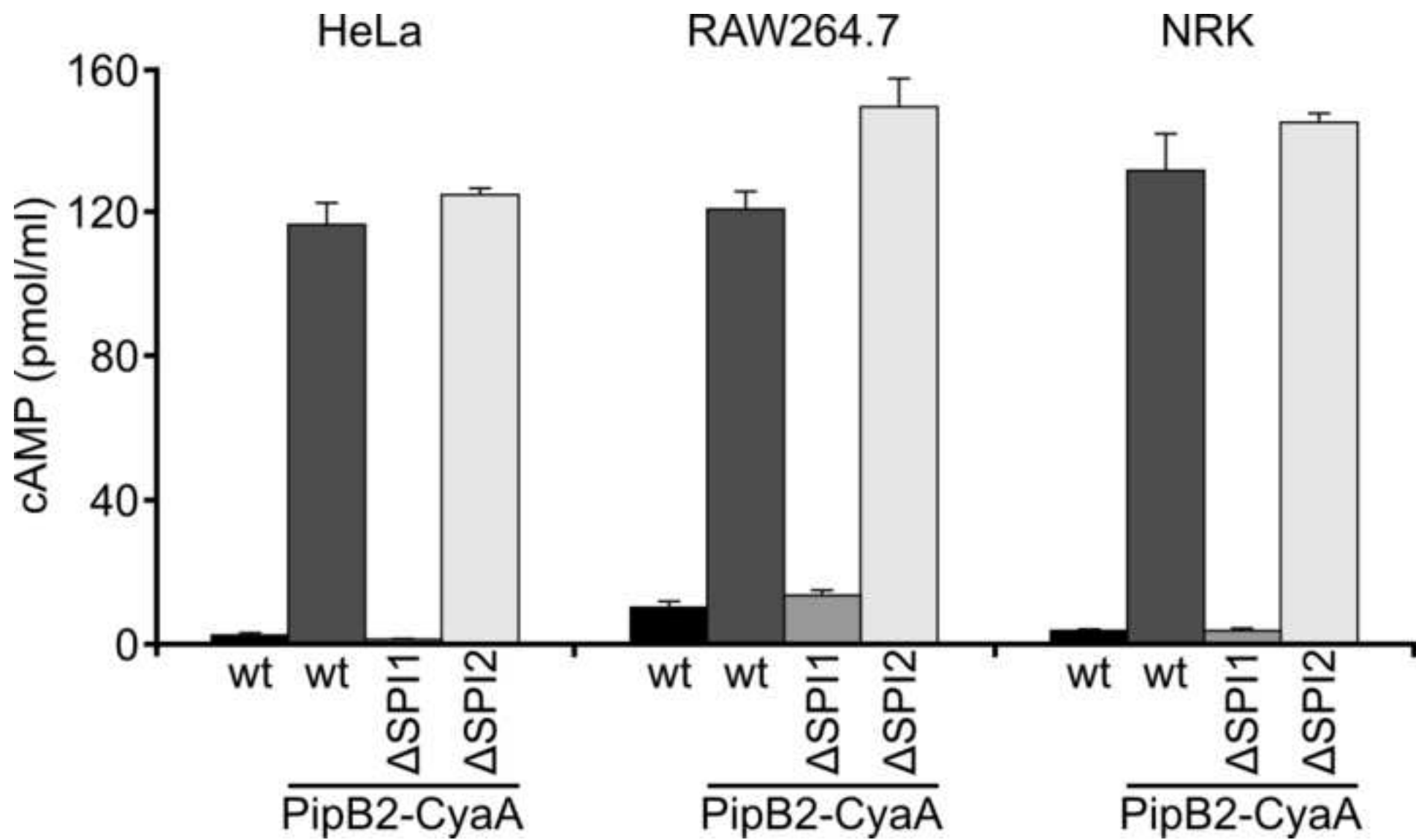
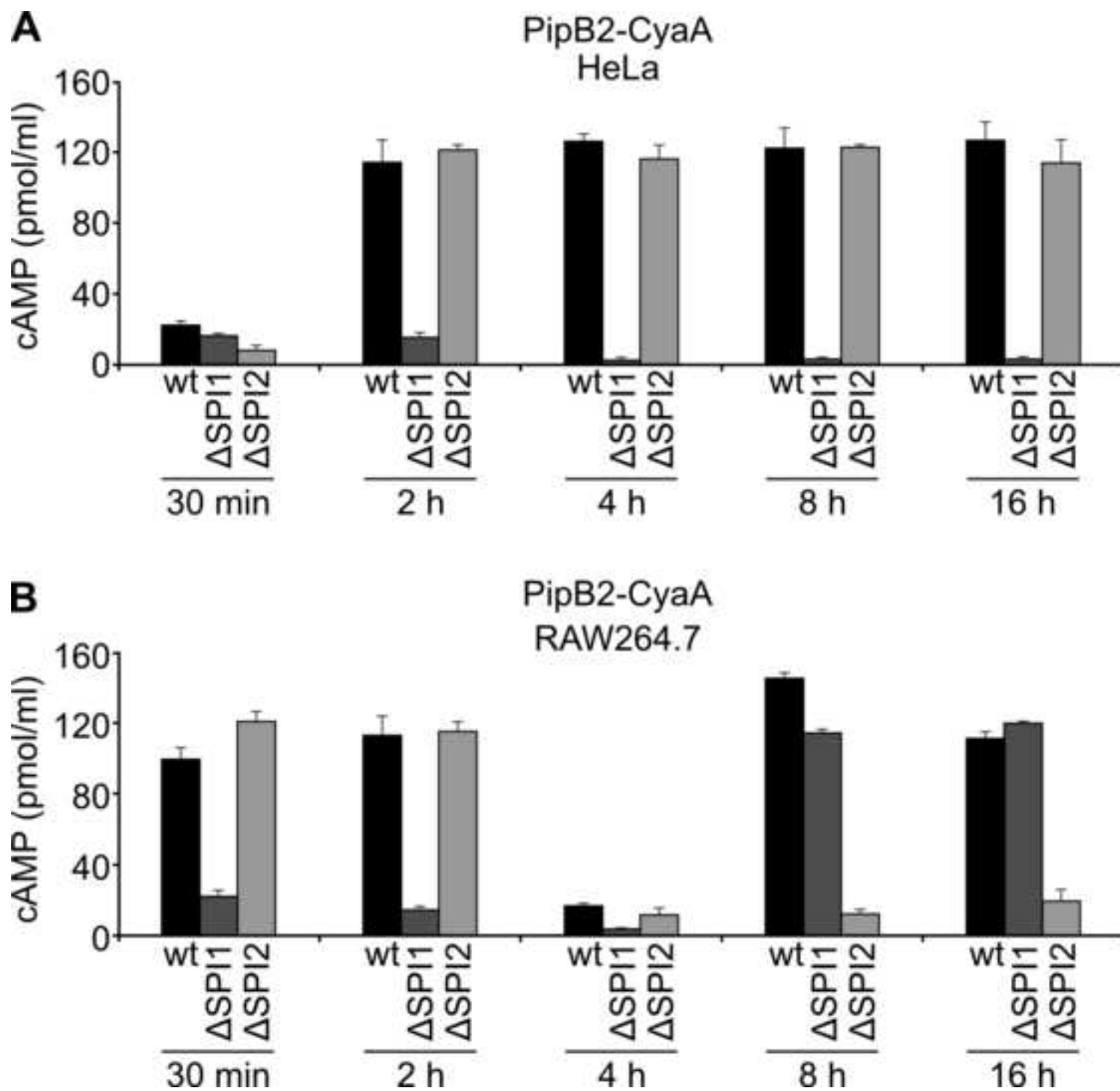


Figure 2 b/w
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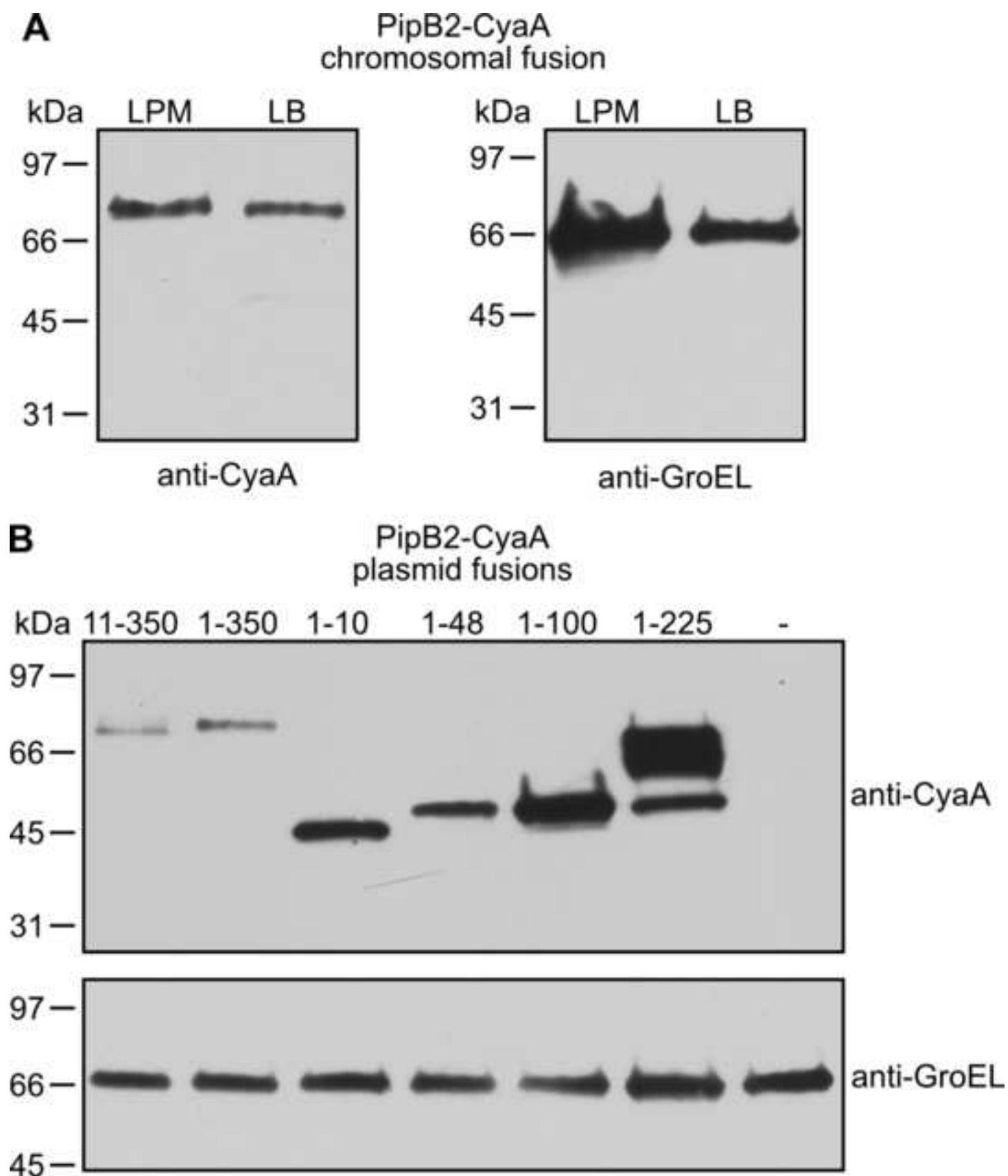


Figure 4 b/w
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