BIOCHEMICAL AND FUNCTIONAL CHARACTERIZATION OF THE BACTERIAL PsaR1 SENSOR IN THE PSEUDOMONAS SYRINGAE PV. ACTINIDIAE-KIWIFRUIT INTERACTION

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INTRODUCTION

Kiwifruit bacterial canker, caused by *Pseudomonas syringae* pv. *actinidiae* (**Psa**), is responsible for important economic losses in all major areas of kiwifruit production worldwide, including Italy (**1**). Innovative plant defense strategies are necessary and should be efficient and eco-compatible, avoiding at the same time the occurrence of new resistance to active molecules. The application of targeted treatments for "weakening" the pathogen, i.e. to reduce its virulence within its host, requires an improvement in our knowledge regarding the molecular mechanisms controlling bacterial virulence induction. The key regulator of bacterial virulence is the so-called 'quorum-sensing' (**QS**) mechanism.
The prototypical **QS** system of Gram-negative bacteria consists of a LuxI-type synthase that produces the signal molecules acyl-homoserine lactones (AHLs) and a cognate LuxR-type receptor/regulator that senses signal specific threshold concentration (**2**). An interesting subgroup of LuxR receptors lacks a genetically linked LuxI and has been termed "solos". These "solos" are assumed to sense AHLs from neighboring bacteria, bacterial molecules other than AHLs or still unknown plant-produced compounds in the case of phytopathogenic bacteria (**3**). Interestingly, Psa does not produce AHLs but possesses three LuxR solos, which likely contribute to Psa virulence (**4**). We are currently investigating the biochemical properties and the possible functions of one of this sensor, namely **PsaR1**.

Expression and Purification

The expression of the full length PsaR1 protein (PsaR1-FL) resulted in the presence of the recombinant protein only in the insoluble fraction (Fig.1). As LuxR proteins are known to be stabilized by the presence of their ligands, we also performed the experiment in presence of kiwifruit macerate or different commercial AHLs added to the growth medium but unsuccessfully since the PsaR1 protein was poorly present in the soluble fraction (Fig.1).
We thus decided to produce a truncated form of the protein containing only the PsaR1 N-terminal domain, the so-called autoinducer binding domain (AIB). Contrary to the full length, PsaR1-AIB was present in the soluble fraction of the lysate obtained after induction (Fig.2) and was thus purified using FPLC (His-tagged protein) (Fig.3).
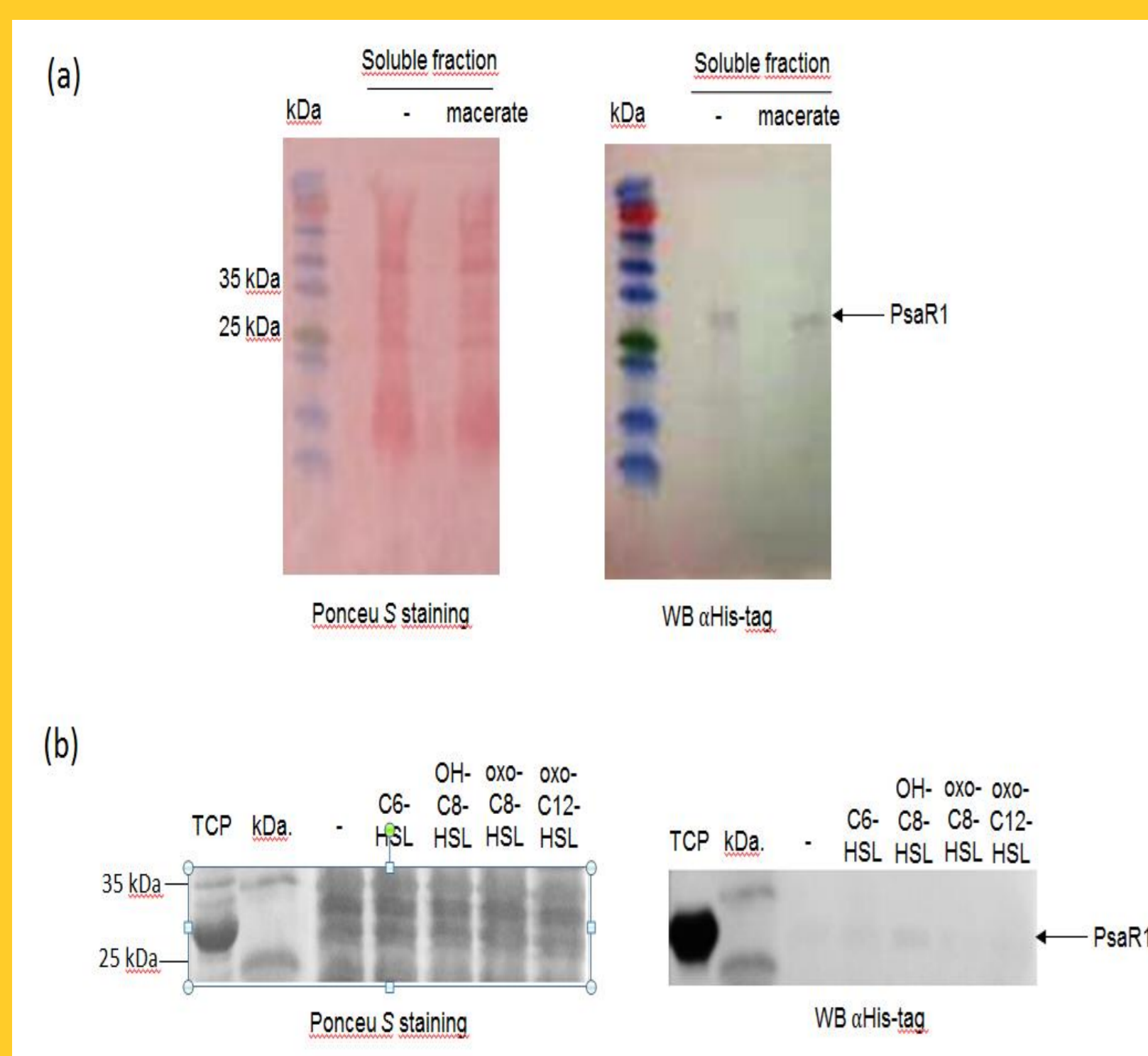
Figure 1. Expression of the recombinant PsaR1-FL (full length) in the soluble fraction in absence or presence of kiwifruit macerate or commercial acyl-homoserine lactones.
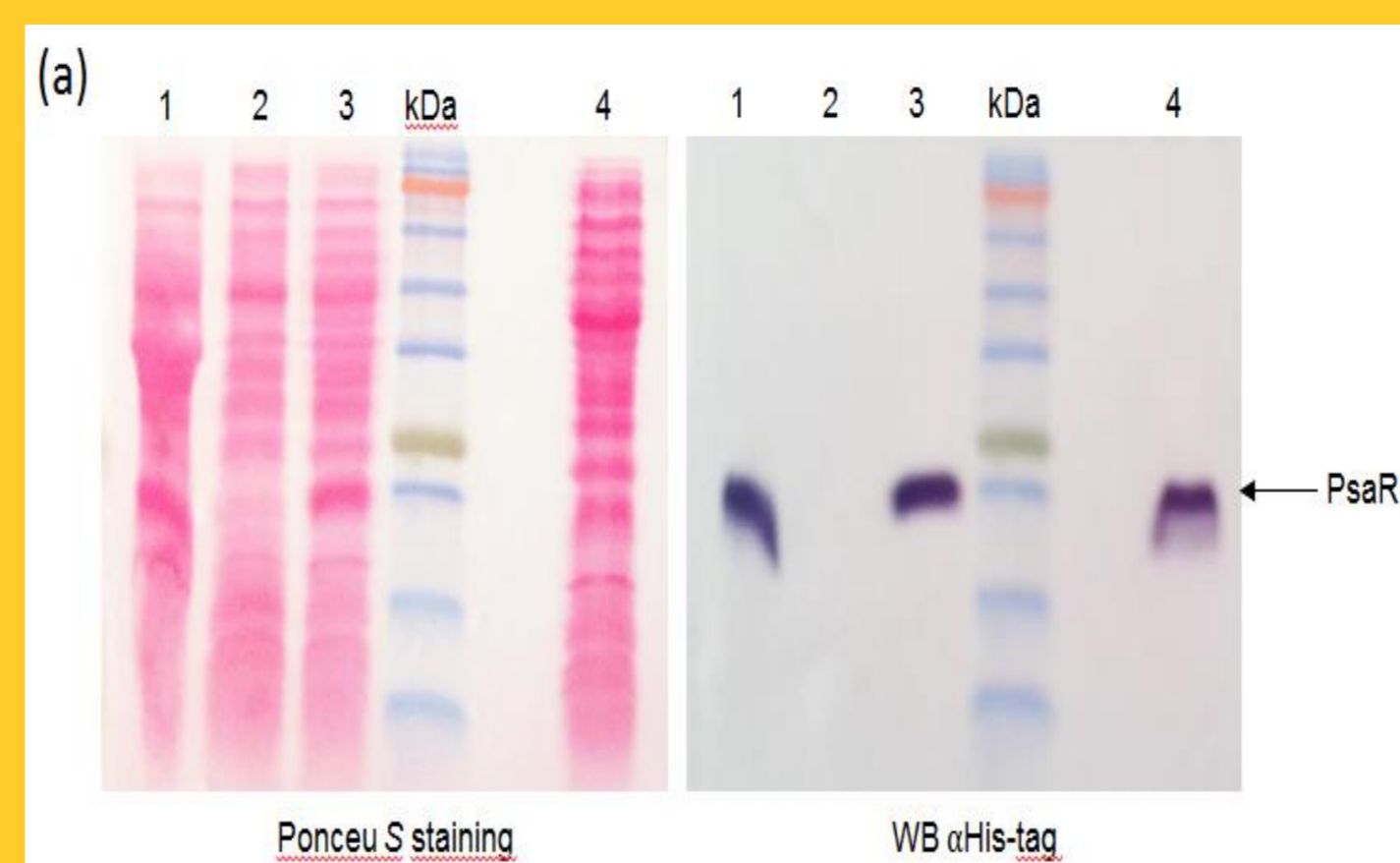
Figure 2. Expression of the recombinant PsaR1-AIB (autoinducer binding domain).
Lane 1: insoluble fraction; Lane 2: total E.coli proteins from Bl21pLysS lysate before induction with IPTG, Lane 3: after induction; Lane 4: soluble fraction.
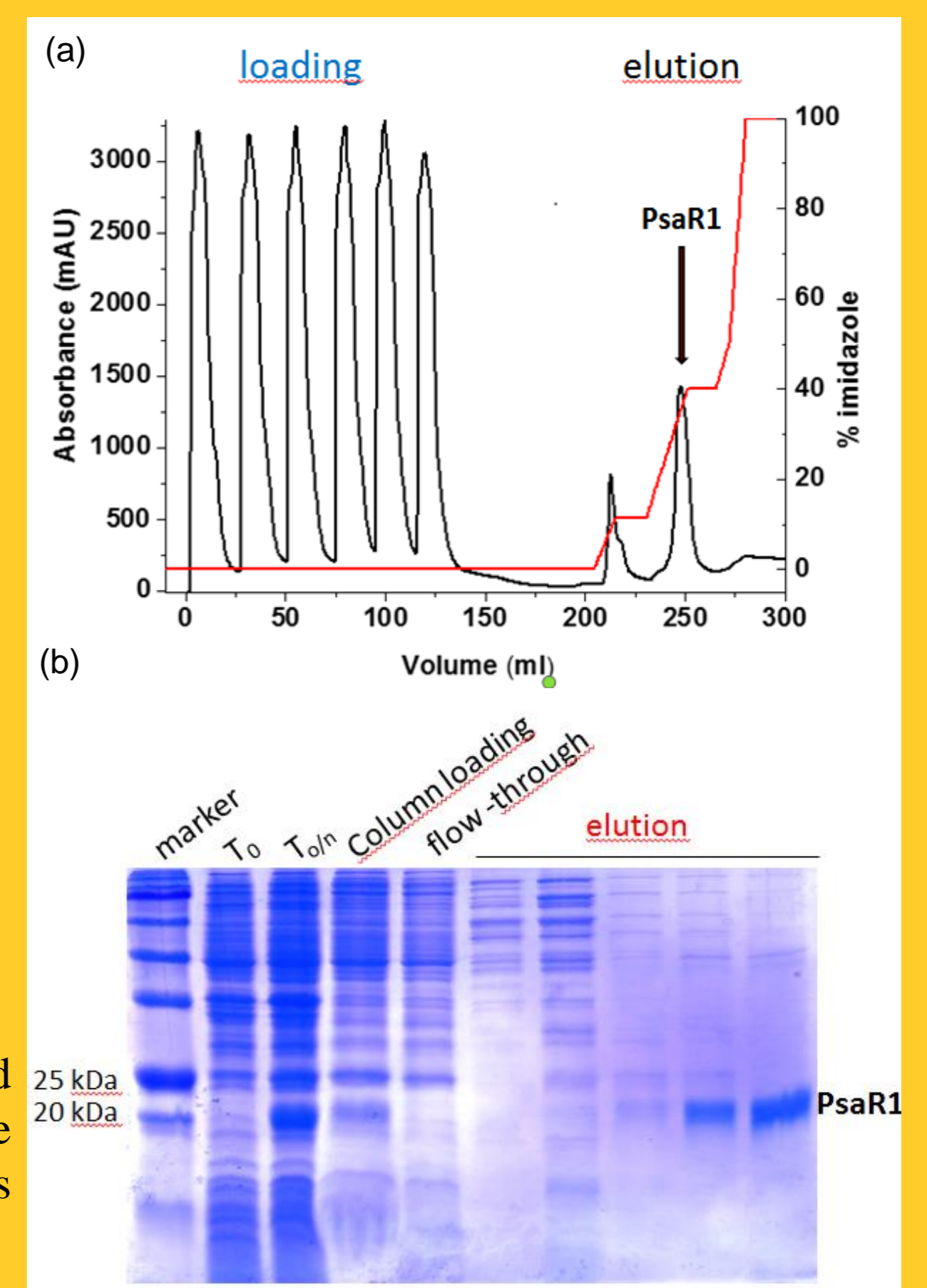

Figure 3. Purification of the recombinant truncated PsaR1-AIB. (a) FPLC elution profile (Nickel-Sepharose column). (b) SDS-PAGE analysis of the fractions obtained in the different steps of PsaR1-AIB purification.

Structural characterization of PsaR1-AIB

Native-PAGE: in absence of DDT the protein PsaR1-AIB is partially aggregated (presence of the recombinant protein in the loading well) and does not migrate properly (Fig.4). By contrast, in presence of DTT, the mobility is more uniform, thus demonstrating that reducing agents are required to ensure the correct folding of the protein.

Size-exclusion chromatography: the SEC analysis of the protein with and without DTT confirmed the previous observations and indicated that PsaR1-AIB is more stable in presence of DTT and tends to form a dimer in these conditions (theoretical monomer MW is 20,86 kDa) (Fig. 5).
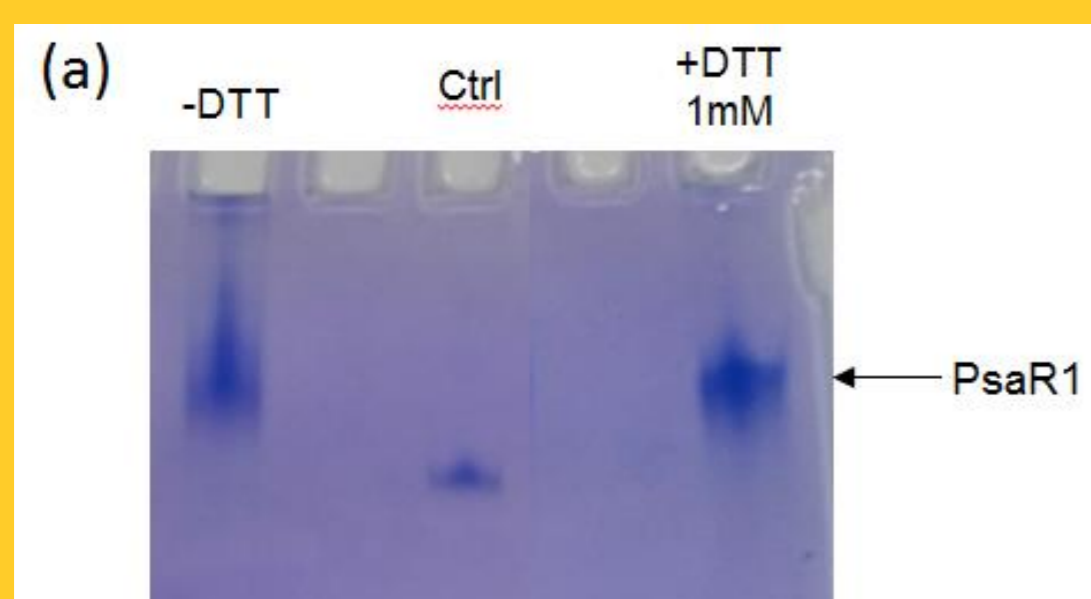
Figure 4. 12% continuous native gel electrophoresis of the purified PsaR1-AIB protein in the absence or presence of the reducing agent DTT (1mM).
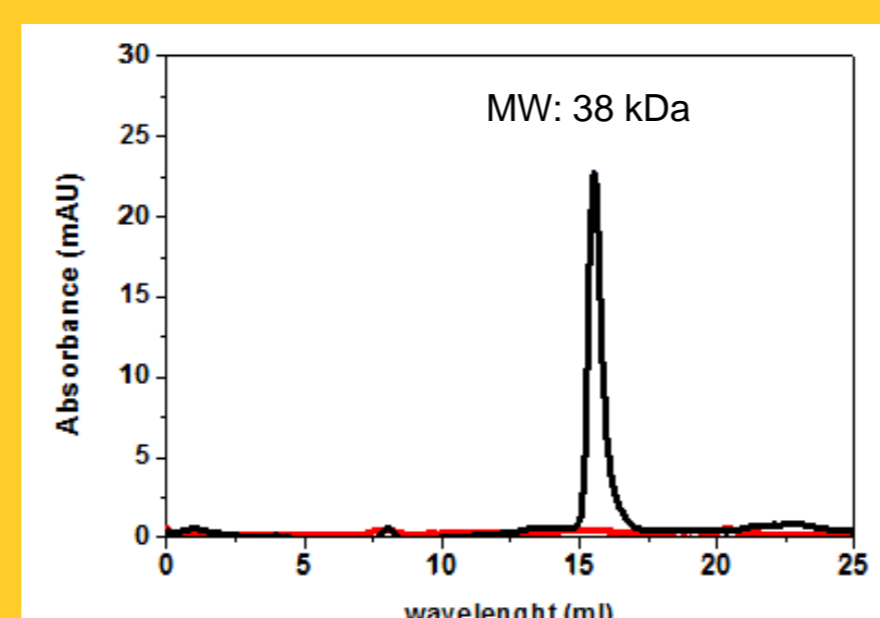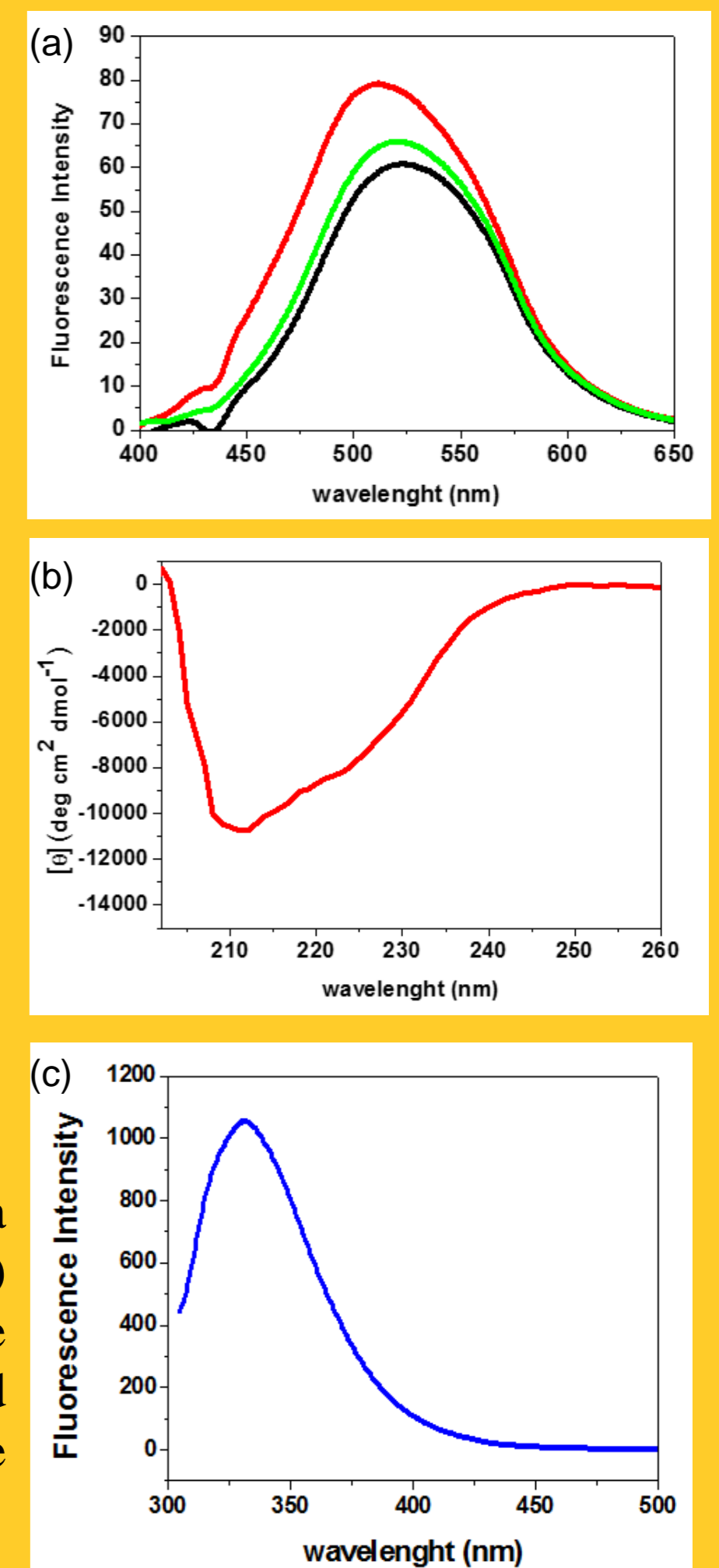
Figure 5. SEC profile of PsaR1-AIB in absence (red) or presence (black) of DTT (1mM). *Calculated dimer MW ~ 38 kDa.*

Binding assay – Thermal Shift Assay

The thermal denaturation profile of the truncated PsaR1-AIB is not affected by the addition of commercial AHLs. These data further confirmed that there is no interaction between PsaR1 protein and the AHLs tested.
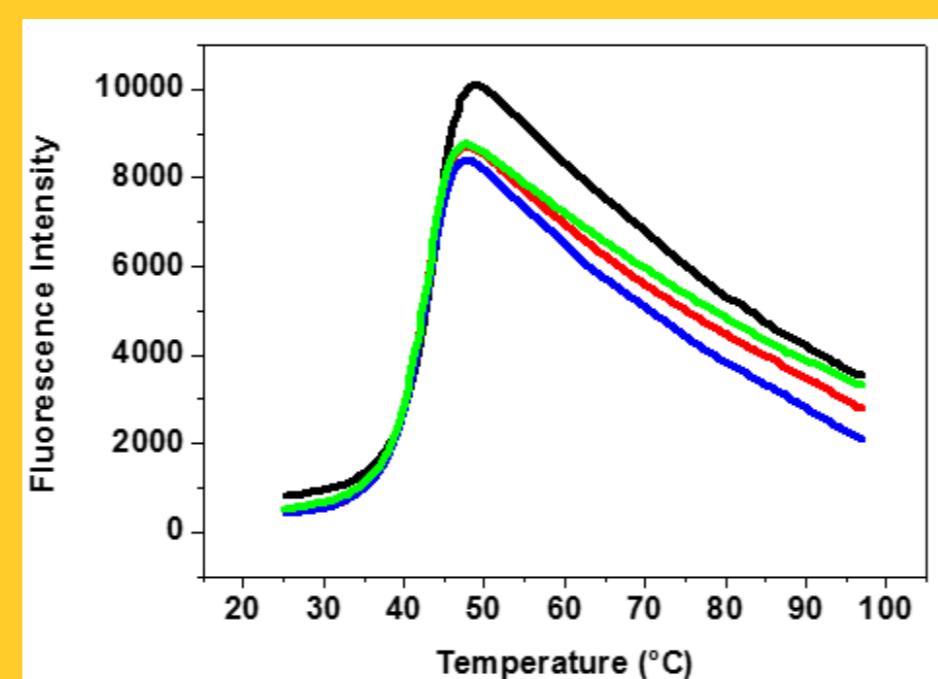
Figure 7. SYPRO Orange fluorescence emission changes as a function of thermal denaturation of the protein in abcence (black) or in the presence (coloured spectra) of different AHL known to be potential ligands of LuxR receptor proteins. The table reports the experimental melting temperatures obtained.

Calculated melting temperatures

protein	Tm °C
PsaR1-AIB	44,1
PsaR1-AIB + C6-HSL	43
PsaR1-AIB + 3-OH-C6-HSL	43
PsaR1-AIB + 3-Oxo-C6-HSL	43

Spectroscopic analysis: ANS (8-Anilino-1-naphthalenesulfonic acid) fluorescence assay showed that, in absence of DDT, the hydrophobic regions of the protein PsaR1-AIB are exposed to the reagent (i.e. to the solvent) (Fig.6a).

The far UV Circular Dichroic spectrum showed that the reduced form of the protein PsaR1-AIB has two strong negative bands with local minima at 208 and 222 nm, typical of proteins with a certain amount of α-helical content (Fig.6b).

Finally, the intrinsic fluorescence spectrum was indicative of a folded protein as the maximum emission of the 3 tryptophan residues within the protein PsaR1-AIB is around 332 nm (Fig.6c).

Figure 6. (a) ANS fluorescence emission spectra (400-650 nm) were recorded for ANS free (black) and for PsaR1-AIB in presence (green) or absence (red) of DTT. (b) Far UV CD spectra of the purified reduced PsaR1-AIB protein. (c) Intrinsic flurescence spectrum of the reduced PsaR1-AIB.
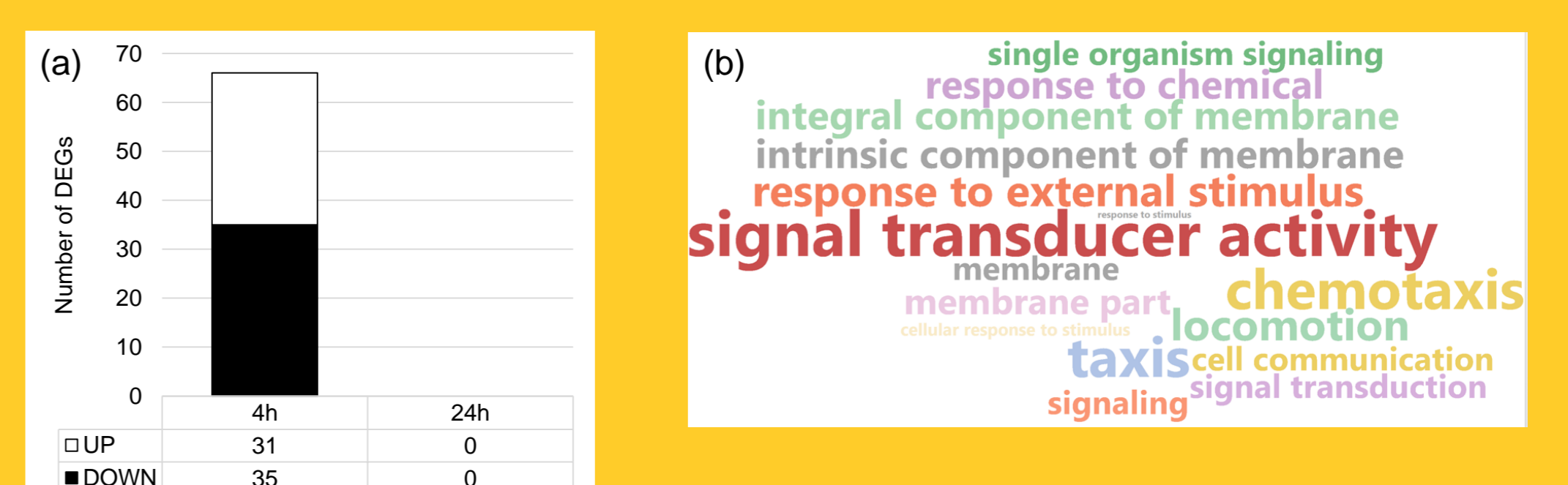
Functional characterization of PsaR1

In order to decipher the putative role of PsaR1 in Psa virulence, we performed a large-scale transcriptomic analysis using a Psa-specific microarray chip designed in our laboratory.
For that purpose, we compared the gene expression profiles of a wild-type Psa strain and its corresponding Δ*psaR1* mutant, both grown in minimal medium, which mimicks apoplast conditions.
We observed that differentially expressed genes (DEGs) were found only after 4h of incubation, indicating that PsaR1 likely plays a role in the early steps of infection (Fig.8a).
Moreover, an analysis of functional category enrichment revealed that the genes down-regulated in the mutant (and thus positively regulated by PsaR1) are mainly related to chemotaxis and signal transduction (Fig.8b).

Conclusions

We successfully expressed and purified a truncated version of PsaR1 containing only the autoinducer-binding domain (PsaR1-AIB). The first analyses revealed that the protein required the presence of reducing agent to ensure its proper folding and thus stability, which results in the formation of a dimer. Moreover, using the recombinant protein, we showed that it does not bind commercial AHLs. This opens the question regarding the putative ligang(s) of the protein. In order to answer to this question, we are currently setting up a high-throughput analysis to apply the thermal shift assay method to the screening of a chemical library. This will allow identifying molecules or at least chemical classes able to bind to PsaR1.
Moreover, a transcriptomic analysis revelaed that PsaR1 may play an important role in the first hours of infection by regulating the expression of genes related to chemotaxis and signal transduction, two key processes of infection allowing the bacterial pathogen to move within its host. Further studies will aim at understanding the mechanisms of regulation of these genes by PsaR1.

	4h	24h
□ UP	31	0
■ DOWN	35	0

Figure 8. (a) Number of differentially expressed genes (up- or down-regulated) in Δ*psaR1* mutant compared to wild-type Psa strain after 4h or 24h of incubation in minimal medium. (b) Functional category enrichment of genes down-regulated in Δ*psaR1* mutant compared to wild-type strain (at 4h in minimal medium).

References
1. Scortichini et al., 2012, Molecular Plant Pathology.
2. Brameyer et al., 2015, Nat Chem Biol.
3. Venturi and Fuqua, 2013, Annu Rev Phytopathol.
4. Patel et al., 2014, Plos One.