

# Overexpression of Plant Specific Insert from Cardosin B (PSI B) in *Arabidopsis* Correlates with Cell Responses to Stresses <sup>†</sup>

Inês Moura <sup>1</sup>, Susana Pereira <sup>1,2</sup>, Ana Séneca <sup>1,2</sup>, José Pissarra <sup>1,2</sup> and Cláudia Pereira <sup>1,2,\*</sup><sup>1</sup> Faculdade de Ciências da Universidade do Porto, Rua do Campo Alegre, s/nº, 4169-007 Porto, Portugal;<sup>2</sup> GreenUPorto - Sustainable Agrifood Production Research Center, Rua do Campo Alegre, s/nº, 4169-007 Porto, Portugal

\* Correspondence: cpereira@fc.up.pt

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**Abstract:** Under abiotic stress, several changes occur in cells both regarding their physiology and cellular mechanisms. Plants have developed modifications in the production and trafficking of proteins and remodeling of endomembranes to overcome stress conditions. The alteration in the targeting of proteins to the vacuole by shifting their transport towards an unconventional, Golgi-independent, route is a good example. Plant Specific Inserts (PSIs) are known to mediate such routes and our goal was to evaluate if transgenic *Arabidopsis* plants overexpressing PSI B respond differently when subjected to different abiotic stresses (osmotic, oxidative, saline, and by metals). The results obtained point to a differential expression of PSI B-mCherry depending on the type of stress and a decrease of cellular and cytoplasmatic movement in all stress conditions.

**Keywords:** Abiotic stress; Plant Specific Insert; protein trafficking; unconventional routes

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## 1. Introduction

Most cultivated plants grow in suboptimal environments, due to abiotic stress factors such as drought, salinity, oxidative stress or metal poisoning [1]. Drought and salinity are the most common abiotic stresses found particularly in crops, and trigger quite complex mechanisms. These type of stresses are associated with a reduction in the osmotic potential of the soil solution resulting in water stress, excess Na<sup>+</sup> and Cl<sup>-</sup> ion toxicity for the cell and nutritional imbalance. It is also related to stomatal closure, decreased photosynthetic activity, altered cell wall elasticity and ROS production [2–4]. Endomembrane trafficking is a fundamental cellular process in all eukaryotic cells, and its regulatory mechanisms have been extensively studied. In plants this process is adjusted quite regularly so that it can adapt to an ever-changing environment. The main endomembrane trafficking pathways in plant cells include secretory and endocytic routes in which the molecular content is transported by vesicles between organelles and is essential for the response and adaptation to abiotic stresses [5,6]. Furthermore, some stresses, such as drought and salinity, play an important role in the process of inducing autophagy, which is quite significant in the tolerance to oxidative stress, a condition associated with most abiotic stresses [7,8]. A recent study in our lab analyzed the expression of several endomembrane-related genes involved in different pathways, in *Arabidopsis* grown under abiotic stress conditions [9]. It was concluded that the pathway to the protein storage vacuole is favoured under stress conditions supporting the putative role of these organelles in plant stress tolerance. The Plant Specific Insert (PSI) corresponds to an independent domain, exclusively found in some plant aspartic proteinases (APs), consisting of about 100 amino acids [10]. Although the biological functions of plant APs are not as well characterized as those of animals, it is known that they have a role in germination, reproduction, senescence, microbial and/or insect defence, protein turnover, programmed cell death and even response to stress [11–

13]. The PSI domain has the ability to interact with membranes and considering that this domain undergoes structural changes depending on pH, the lipid composition of membranes govern these interactions [14]. When isolated and in vitro, this domain has a wide range of functions such as antimicrobial activity, permeabilization and membrane modulation, secretion of vesicular contents, protein processing and also functions as a vacuolar sorting signal [15–18]. It has been demonstrated that the PSI domains present in cardosins A and B (two APs isolated from cardoon—*Cynara cardunculus* L.) and in AP1 and AP2 (APs isolated from Soybean) are capable to redirect secreted proteins to the vacuole through different pathways [19]: Glycosylated PSIs mediate vacuolar transport dependent on COPII vesicles and, therefore, following the conventional route, while non-glycosylated PSIs mediate the transport in a Golgi independent manner. These two pathways seem to provide a certain resilience to plant cells as they can be activated depending on the stage of development and environmental conditions [17,19,20]. Taking this into account, the general objective of this work was to study the expression levels of PSI B from cardoon and its biosynthetic pathways in transgenic *Arabidopsis* plants overexpressing this domain coupled to m-Cherry fluorescent protein, in physiological and under abiotic stress conditions. Our results show that PSI B expression is correlated with some stress conditions, although its intracellular localization remained unchanged. Interestingly, subcellular movements observed in root cells expressing PSI B-mCherry are dramatically decreased under stress conditions.

## 2. Material and Methods

### 2.1. Plant Material and Stress Assays

Homozygous PSI B-mCherry lines were obtained using the floral-dip method [21] and tested for the presence of the transgene by PCR and Western Blot (data not shown). Seed germination was performed in MS (Mourashige and Skoog—Duchefa) medium with 1, 5% (*w/v*) sucrose and supplemented with the stress-inducing agents as described previously [9]. In short, in the abiotic stress tests different conditions were used: S1 and S2 (salinity)—sodium chloride (50 mM and 100 mM, respectively), mannitol (50 mM and 100 mM), hydrogen peroxide (0.5 mM) and zinc sulfate (150  $\mu$ M), respectively, to the environment already described.

### 2.2. Root Biometrical Analysis

A phenotypic study was done to evaluate biometric parameters. For this assay, the seedlings were germinated in the conditions already described, but square plates were used and incubated vertically. After a period of ten to twelve days the plates were collected, analyzed and a photographic record was done. Using ImageJ/Fiji software, a metric analysis of the roots was obtained in two conditions: wild type *Arabidopsis* plants and *Arabidopsis* overexpressing PSI B.

### 2.3. cDNA Preparation

To obtain total RNA preparations, 50 mg of seedlings were weighted and the “NZY Total RNA Isolation Kit” (NZYTech) was used according to the manufacturer instructions. Using a microdrop spectrophotometer (DeNovix DS-11, Bonsai Lab), total RNA was quantified, and its integrity was verified in a 1% agarose gel. Total RNA was then stored at  $-80^{\circ}\text{C}$ . The cDNA preparation from RNA samples was performed using the “NZY First Strand cDNA Synthesis Kit” (NZY Tech) following the protocol provided.

### 2.4. Quantitative RT-PCR

Three technical replicates were performed for each gene and condition, in 10  $\mu$ L final reaction volume including 400 nM of each primer and 2  $\mu$ L of cDNA, diluted eight times. The quantitative RT-PCR was done using PowerUp SYBR Green Master Mix (Thermo Fisher) and it was performed in a CFX96 Real-Time System (Biorad). The PCR reaction

was conducted as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, 56 °C for 10 s and 72 °C for 30 s. After 40 cycles, and to generate the melt-curve, the following conditions were used: 95 °C for 10 s, followed by a constant increase from 65 to 95 °C. Table 1 list the primer pairs used for each gene. The SAND-1 and UBC9 genes were used as reference genes [22].

**Table 1.** List of genes and corresponding primer pairs used in the quantitative RT-PCR assay.

Gene	Primer Forward	Primer Reverse
UBQ_9	TCACAATTTCCAAGGTGCTGC	TCACAATTTCCAAGGTGCTGC
SAND-1	AACTCTATGCAGCATTGATCCACT	TGATTGCATATCTTTATCGCCATC
m-Cherry	GACCACCTACAAGCCAAG	GTGGGAGGTGATGTCCAAC

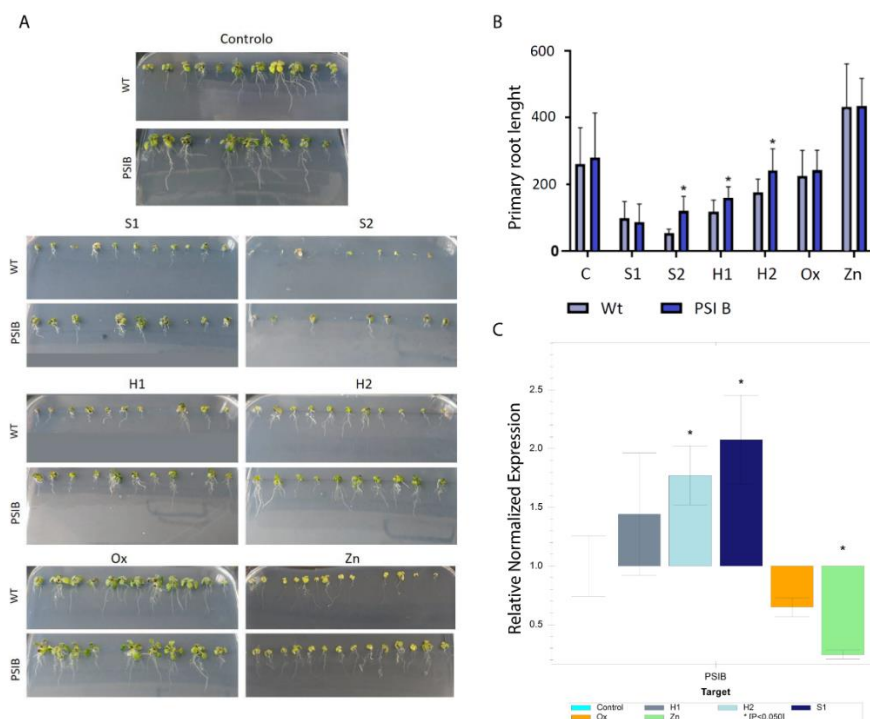
### 2.5. Confocal Microscopy

Arabidopsis seedlings expressing PSI b-mCherry grown under the stress conditions referred above were imaged using Confocal Laser Scanning Microscopy (CLSM, Leica Stellaris 8). mCherry emission was detected between 580–630 nm, using 561 nm excitation. Images were processed using ImageJ/Fiji software. For the mean intensity quantification, the same settings were used to acquire the images and more than 20 cells were quantified.

## 3. Results

### 3.1. PSI B Expression Changes in Plants under Abiotic Stress

After obtaining homozygous PSI B-mCherry lines, and to study the influence of different types of stress on the expression of PSIs of *Arabidopsis thaliana*, abiotic stress tests were performed. Regarding the morphological aspects of the seedlings, in the lower salt concentration (S1) and high concentration water stress (H2) the changes were quite identical, with a noticeable delay in development and some leaf chlorosis (Figure 1A).



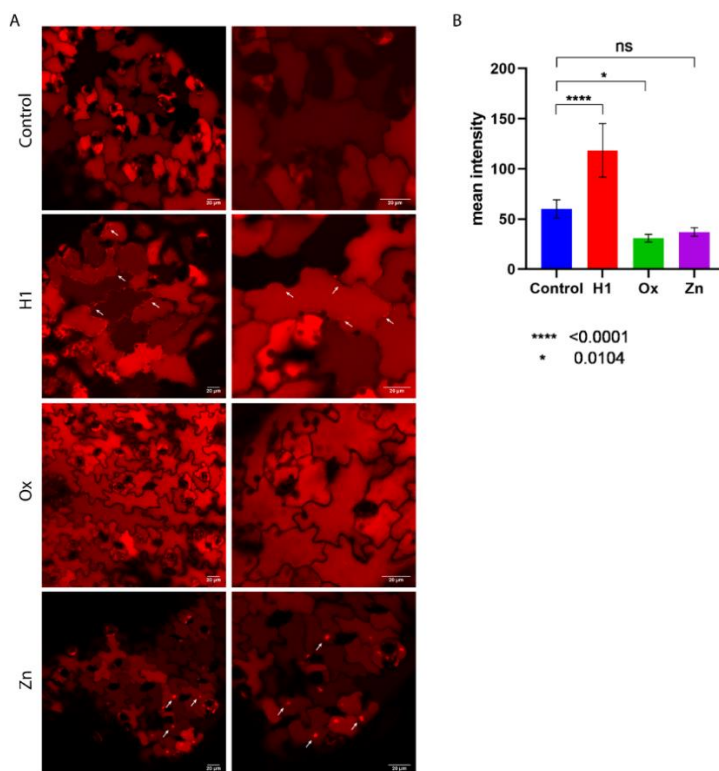
**Figure 1.** Phenotypic and expression analysis of *Arabidopsis* plants expressing PSI B-mCherry grown under stress conditions. (A)—*Arabidopsis thaliana* germinated for 10 to 12 days in stress conditions. (B)—Primary root metric analysis. (C)—PSI B-mCherry expression analysis by qPCR rela-

tive to Control. \*—Statistically significant values. S1—Low salt concentration; S2—High salt concentration; H1—Mild water stress; H2—High water stress; Ox—Oxidative Stress; Zn—Stress induced by metals.

The plants in the mildwater (H1) and metal stresses (Zn) showed a slight delay in development in relation to the control and those in Zn showed a high degree of chlorosis. Regarding oxidative stress (Ox) the plants do not show any morphological changes. In the high salt concentration (S2) the plants did not develop and, therefore, this condition was not used for further evaluations. Analysis of the primary root length of wild type and *Arabidopsis* plants overexpressing PSI B-mCherry, in the same stress conditions, was performed (Figure 1B). In the control conditions no changes were visible. In S1, both in wild type and transformed plants, it was verified a markedly decrease in root development, with similar values in control and PSI B plants. However in S2, and despite the plant development being quite compromised, it is noticeable a significant increase in the root length of plants expressing PSI B-mCherry when compared to WT plants. The same tendency can be observed in plants grown under H1 and H2 stresses. Regarding the plants under Ox and Zn conditions no changes are observed compared to the control. The expression of PSI B-mCherry in *Arabidopsis* grown under abiotic stress conditions was evaluated by qPCR, using the normalized expression method, with a  $p$ -value of 0.05 and the results presented are relative to control conditions (Figure 1C). From the data obtained, PSI B-mCherry is upregulated in H1, H2 and S1 grown plants, despite only H2 and S1 with significant values. Interestingly, in Ox and Zn grown plants the situation is reversed and PSI B-mCherry is downregulated.

### 3.2. PSI B Localisation in *Arabidopsis* Plants under Stress

Given the changes in PSI B-mCherry expression and the phenotypes observed, we checked whether the localisation of PSI B was altered in leaves and roots of plants grown in H1, Ox and Zn stress conditions when compared to the control (Figures 2 and 3).

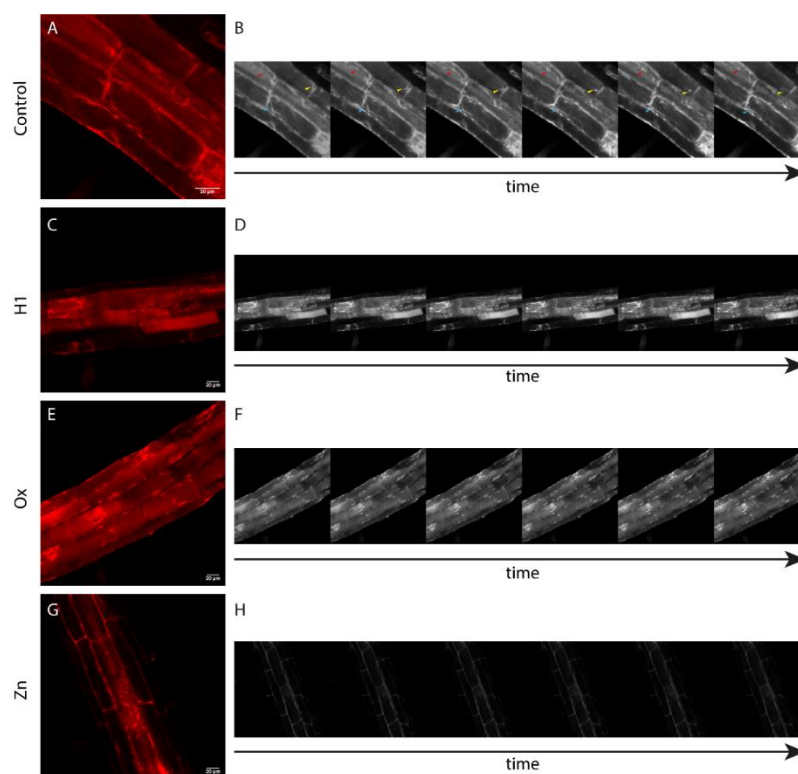


**Figure 2.** Confocal microscopy analysis of leaves from *Arabidopsis* plants expression PSI B-mCherry, under stress conditions. (A)—Representative images of leaf cells. Arrows indicate devi-

ation patterns relative to control. (B)—Quantification of the mean fluorescent intensity in cells expressing PSI B-mCherry. \*—Statistically significant values. H1—Mild water stress; Ox—Oxidative Stress; Zn—Stress induced by metals.

In leaves (Figure 2), PSI B-mCherry accumulates mainly in the vacuole in all the conditions studied (Figure 2A), despite some differences in the fluorescence intensity were detected, as shown by the mean fluorescence quantification (Figure 2B). A significant higher amount of fluorescence was detected in the leaf cells of plants grown under H1 condition, while a decrease in fluorescence can be observed in those grown under Ox condition. Interestingly, in the H1 conditions, some fluorescence can be observed at the periphery of the cell (Figure 2A, H1-arrows), resembling Endoplasmic Reticulum patterns. In the Zn condition, it is possible to detect some fluorescent agglomerates inside some cells (Figure 2A, Zn-arrows) that are not observed in any other condition.

In root cells, the accumulation pattern for PSI B-mCherry is quite different from that of the leaves (Figure 3). In control plants, PSI B-mCherry accumulates around the nucleus and, at the cell periphery, in compartments that move within the cell (Figure 3B, coloured arrowheads; Movie S1). Interestingly, in the other conditions tested although PSI B-mCherry fluorescence is still detected in similar compartments (Figure 3C,E,G) the movements are no longer detected (Figure 3D–F; Movies S2–S4). Additionally, in H1 condition, several cells presented some fluorescence in the vacuole (Figure 3C) and in Ox grown plants fluorescence aggregates can be found inside the cells (Figure 3E).



**Figure 3. Confocal microscopy analysis of roots from *Arabidopsis* plants expression PSI B-mCherry, under stress conditions. (A,C,E,G)—Representative images of root cells expressing PSI B-mCherry. (B)—Stills retrieved from time-lapse video illustrating the movement of PSI B-mCherry-labeled compartments (colored arrows). (D,F,H)—Stills retrieved from time-lapse video illustrating the movement of PSI B-mCherry-labeled compartments. H1—mild water stress; Ox—Oxidative Stress; Zn—Stress induced by metals.**

#### 4. Discussion

Throughout evolution, plants are exposed to adverse environmental conditions, and to face and respond to these stresses plants developed special mechanisms of cellular reorganization. Most abiotic stresses have similar physiological consequences, such as the induction of cell damage, and thus inducing similar signalling pathways. In this work, the conditions in which *Arabidopsis* plants were grown not only caused changes at the morphological level but also in the expression of PSI B. A recent study regarding APs expression suggested their involvement in defence or tolerance to hydric and salt stress [23]. In similar conditions our results show a higher expression of PSI B. The observed higher fluorescent accumulation of PSI B-mCherry in the H1 grown plants supports those results. The downregulation of PSI B-mCherry on metal and oxidative stresses reinforces the importance of this domain in hydric and saline stresses, and may indicate a role in processes related to cellular homeostasis and water control. A study performed by our team [24] regarding *Arabidopsis thaliana* aspartic proteinases revealed that, depending on the abiotic stress, the three APs genes tested had different and antagonistic expression levels. In plants grown under lower salt concentration PSI B-mCherry expression behaves similarly to the overexpression of AP1 and AP3. Likewise, in the heavy metal stress assay the downregulation of PSI B resembles the behavior of AP3. This similarity may be related to AP3's function, being implicated in the processing and degradation of storage proteins. This is quite interesting since this overexpression in salt and mild hydric stresses, suggests that degradation of storage proteins occurs to overcome and tolerate these restricted conditions. Based on these results, we can infer that PSI B may have a function related to the activation of genes responsible for the degradation of storage proteins. In addition, the study also showed the expression of several endomembrane transport-related genes involved in different vacuolar pathways in response to abiotic stress [9,24]. The results revealed that the pathway to the protein storage vacuole is enhanced, and since PSI is a vacuolar determinant [17], it may participate in this reinforcement phenomenon. Thus, the PSI may then be associated with development and the defence system, and yet operate by distinct mechanisms, as each type of stress may trigger a different response [9,14]. Regarding the biometric parameters, and particularly the root length, it seems that the expression of PSI B may also influence the plant ability to face some adverse conditions. Looking at high salt conditions, there is a significant increase in the root length in the transformed plants, suggesting an increased stress tolerance provided by PSI B overexpression that may be triggered at certain concentrations. The plants grown under high and mild water stress conditions also showed significant changes in roots length. Given the PSI roles in membrane interaction or protein processing [15,18], these results suggest increased tolerance in order to mitigate the negative effects of environmental adversities. Interestingly, analysis of PSI B-mCherry localization in roots showed a marked decrease in cytoplasmic movements under all stress conditions, raising the question whether vesicle movement can be constrained by stress.

#### 5. Conclusions

In the present study, we analyzed the expression of PSI B during stress conditions. From the results obtained we can suggest that PSI B has an active role in adaptation and tolerance mechanisms against abiotic stress particularly in salt and hydric stress, where its expression has a positive effect on plant fitness. Despite still being very preliminary, the data presented here is encouraging and it is worth investigating in higher detail the role of the PSIs, and aspartic proteinases in general, in plants responses to stress.

**Supplementary Materials:** The following are available online at [www.mdpi.com/xxx/s1](http://www.mdpi.com/xxx/s1), Movie S1: Time-lapse video illustrating the movement of PSI B-mCherry-labeled compartments in control conditions, Movie S2: Time-lapse video illustrating the movement of PSI B-mCherry-labeled compartments in H1 condition, Movie S3: Time-lapse video illustrating the movement of PSI B-mCherry-

labeled compartments in Ox condition, Movie S4: Time-lapse video illustrating the movement of PSI B-mCherry-labeled compartments in Zn condition.

**Author Contributions:** I.M., A.S., S.P. and C.P. conceived and designed the experiments; I.M. performed the experiments; I.M., A.S. and C.P. analyzed the data; J.P. contributed reagents and materials; I.M. and C.P. wrote the paper; A.S, S.P. and J.P. reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

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