

# Abiotic Stress Upregulates the Expression of Genes Involved in PSV and Autophagy Routes <sup>†</sup>

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**Abstract:** Adverse conditions caused by abiotic stress modulate the plant development and growth by altering morphological and cellular mechanisms. To face this problem, plants, along with physiological adaptations, developed intracellular mechanisms, including changes in protein production and trafficking or modifications of the endomembrane system. It is known that stress situations can alter protein sorting to the vacuole, changing their routes via a Golgi-independent pathway. Our goal is to evaluate the expression levels of different aspartic proteinases and well-characterized genes involved in the vacuolar pathway, in plants submitted to different abiotic stresses (osmotic, oxidative, saline and heavy metals). The results obtained point to a different response of the three aspartic proteinases under study, indicating that different, yet related, genes respond differently to different types of stress, resulting in a fine-tuned regulation. Furthermore, our results regarding the endomembrane system effectors show that EXO70, RMR1, SYP51, SYP121 and VTI12 are up regulated, while VAMP, SYP23 and BP80 are downregulated in the same situations. This demonstrates that adverse conditions caused by abiotic stress can alter the expression of key proteins involved in the protein trafficking machinery, which can be related with the activation/deactivation of certain pathways.

**Keywords:** abiotic stress; aspartic proteinases; endomembrane trafficking

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## 1. Introduction

Climate change stands, nowadays, as the foremost threat to human health, causing crop failures worldwide and leading to food insecurity [1]. Due to their limited locomotion, plants evolved the ability to adapt to, and take advantage of, changes in climate and environment [2]. Plant stress can be defined as any environmental condition that does not allow the plant to reach its full genetic potential. Abiotic stress is the main cause of the loss of quality and productivity for crops in the world. Drought, salinity, temperature and oxidative stress are often interrelated and cause similar cellular damage [3]. These diverse environmental stresses often activate signals and pathways involved in similar cellular responses: overexpression of antioxidants, accumulation of solutes, changes in protein trafficking and endomembrane remodeling [4–6]. In recent years using high throughput screening techniques, such as microarrays and RNA sequencing, it was possible to identify many stress-related genes. These techniques provide us with important information and suggest genes among which it is possible to identify new markers for assisted selection of varieties resistant to stress. The change in the transcriptome is still the result of a complex series of events and understanding the mechanism of stress response is only partial. The identification of the specific roles of each actor in the game turns out to be an important factor for the genetic improvement of plants because the

positive adaptation probably depends on synergistic effects and balanced interactions among proteins normally not related [7]. Recent experimental evidence [7,8], suggests that several classes of proteins (like Aquaporins, SNARES, ATPase pumps or channels) can control specific events of membrane transport leading to important events of cell reorganization in adverse environmental conditions. Several research groups found interesting connections between stress tolerance and membranes rearrangements not observed before as it was the case of a potassium channel selectively accumulated on small vacuoles not observed before [9] and sufficient to confer stress tolerance when overexpressed. Nonetheless the connection between the structural architecture of membranes and stress tolerance is not sufficiently investigated. In the last decade, the outburst of data on molecular mechanisms involved in protein trafficking have outlined subtle balances between the transport routes [10,11]. The physiology of plants, and crops in particular, subjected to stress has been the aim of several studies through the last decades, but none or little attention has been given to the molecular mechanisms controlling these rearrangements.

Transport and compartmentalization events of large amount of material out of the classic vesicle traffic route have escaped full characterization. One example is the recently characterized vacuolar route mediated by the PSI (Plant Specific Insert), that was shown to follow an alternative pathway, independent of the Golgi [12]. The PSI is a unique domain, with approximately 100 amino acids, present in the primary structure of some plant aspartic proteinases (APs) [13,14], which plays a role in germination, senescence, organism defense and protein turnover [15] This sequence is responsible, before being excised, for the sorting of these APs to the protein storage vacuole (PSV) and to protein bodies, but when isolated it has the capacity to sort other proteins to the vacuole, being considered an unconventional vacuolar sorting domain [16]. When associated with proteins, PSI can also determine their intracellular pathway depending on PSI's glycosylation status, in other words, PSI mediate a conventional pathway when glycosylated, but will follow a route, directly from the ER to the Golgi, when is not glycosylated [12]. This process is generically indicated as unconventional trafficking, and several examples have been described [10,17–19]. Many of these unconventional routes are associated with development and stress-induced conditions, since cells need to adapt their trafficking machinery to new, challenging, scenarios.

In the context of rapid global climate change and growing production demands are compatible with sustainable agriculture, so it is necessary to develop new selection markers that are part of more complex interactomes that, so far, escaped phenotyping. In this context, this work aims to contribute to the understanding of mechanisms of reorganization and remodeling of plant cell endomembranes in response to abiotic stress. We evaluated the expression of three aspartic proteinases from *Arabidopsis thaliana* along with several well-known endomembrane system effectors, in response to different types of abiotic stress, by RT-qPCR. Our results show that some genes are upregulated when plants grow in adverse conditions, while others are downregulated, indicating a positive regulation of some trafficking routes.

## 2. Experiments

### 2.1. Biological Material Preparation

*Arabidopsis thaliana* (col0) seeds were germinated in Murashige and Skoog medium (MS) (Duchefa), supplemented with 1.5% (w/v) sucrose and 0.7% (w/v) bactoagar. To simulate the abiotic stress conditions, to this medium were added different concentrations of sodium chloride (saline stress: S1–50 mM and S2–100 mM), mannitol (hydric stress: H1–50 mM and H2–100 mM), hydrogen peroxide (oxidative stress: Ox–0.5 mM) and zinc sulfate (heavy metal-induced stress: Zn–150  $\mu$ M). Plates with seeds were maintained in a growth chamber for ten to twelve days, at 21 °C with a photoperiod of 16 h light and average humidity between 50 and 60%. Three biological replicates were prepared.

### 2.2. cDNA Preparation

Total RNA preparations were obtained from 100 mg of seedlings, using the “GeneJET Plant RNA purification Mini Kit” (ThermoFisher) according to the manufacturer instructions. Total RNA was quantified using a Nanodrop spectrophotometer (DeNovix DS-11, Bonsai Lab) and its integrity was verified in a 1% agarose gel. cDNA was obtained from 2.5 µg of total RNA using *SuperScript IV VILO Master Mix* (ThermoFisher), following the protocol provided. Both RNA and cDNA preparations were kept at -80 °C until use.

### 2.3. Quantitative RT-PCR

The quantitative RT-PCR was performed in a CFX96 Real-Time System (Biorad) using PowerUp SYBR Green Master Mix (Thermo fisher). Three biological replicates and three technical replicates were performed for each gene and situation, in 10 µL reaction volume including 400 nM of each primer and 2 µL of cDNA, diluted eight times. The primer pairs used for each gene are listed in Table 2. The amplification conditions were as follows: initial denaturation (95 °C for 3 min) followed by 40 cycles of amplification and quantification (95 °C for 10 s, 56 for 10 s and 72 °C for 30 s, with a single fluorescence measurement) and melting curve generation (65 °C to 95 °C with one fluorescence read every 0.5 °C). Calculation of cycle threshold (Ct) and primer efficiency was performed using Bio-Rad CFX Maestro (version 1.0) software. The analysis of the results obtained was made by relative quantification, using the same software, and by comparison with the control situation. The housekeeping genes SAND-1 and UBC9 were used as reference genes [20].

**Table 2.** List of genes and corresponding primer pairs used in the quantitative RT-PCR assay.

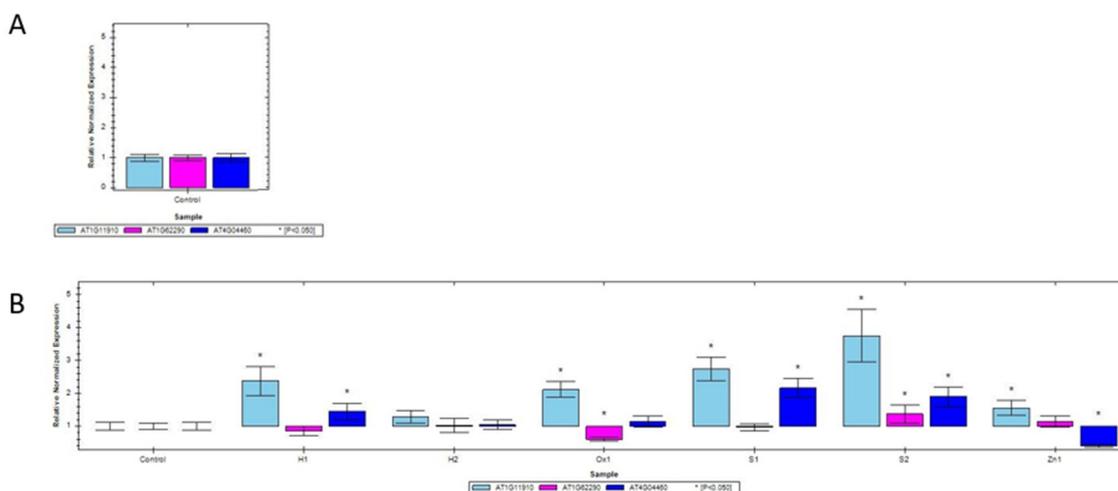
Gene	Primer Forward	Primer Reverse
<i>At1g11910</i>	GGCATTGAGTCGGTGGTGGACA	TCTCACATGCAGAACACGCAGCA
<i>At1g62290</i>	GGGGATTGAATCGGTGGTGGGA	ACATGCAGGACAACCCGCGTCT
<i>At4g04460</i>	TGCAAGGCCGTGGTGGATCA	GCGCAGACTCCAATTTGTGAGCA
<i>AtSYP 23</i>	GCAGCGTGCCCTTCTTGTGG	TCCTTGGGCAGTTGCAGCGTA
<i>AtSYP 121</i>	TCCTCCGATCGAACCCAGGACCTC	TTCTCGCCGGTGACGGTGAA
<i>AtVAMP723</i>	CCCGTGGTGTGATATGTGAG	CCACAAACCGAGAGGATGAT
<i>AtVTI 12</i>	GCAATGTCCGTGGAGAGGCTTGA	TGCGCATGAAGGAGGGTTTGG
<i>AtBP-80</i>	GGGAGCGGCGCAGATTCTTG	GCCGGTTTCATTTCGCCACCTT
<i>AtRMR1</i>	GCGAGGGAGGCACACCAGGA	TTCCCCGGCCTTGTGGTGA
<i>AtEXO-70</i>	TCCCCGATGAAACAGGCTCGTC	GCCTCCATGAAAGGGGCGTGT
<i>UBC9</i>	TCACAATTTCCAAGGTGCTGC	TCATCTGGGTTTGGATCCGT
<i>SAND-1</i>	AACTCTATGCAGCATTGATCCACT	TGATTGCATATCTTTATCGCCATC

## 3. Results

### 3.1. Expression of *A. thaliana* Aspartic Proteinases under Stress Conditions

Three different, yet related, AP genes from *Arabidopsis thaliana* were selected for this study - *At1g11910* (AP1), *At1g62290* (AP2) and *At4g04460* (AP3). The relative expression of each gene was analysed by qRT-PCR in plants germinated under stress conditions. Comparing the expression patterns of the three APs genes relative to 0, is clear that their expression in control situations is similar (Figure 1A). In order to compare the alterations in the expression patterns of these genes in the different types of stress, the results were analysed relative to the control situation, and the genes grouped relative to each stress condition (Figure 1B). In the saline stress, AP1 and AP3 are overexpressed in the two concentrations, while AP2 is only overexpressed in the higher concentration (Figure 1B, S1 and S2). Nevertheless, the expression of AP1 is always higher, in this condition (Figure 1B, green bars). The hydric stress shows similar results in the lower concentration, while in the higher concentrations there are not significant changes in the expression (Figure 1B, H1 and H2). Again, AP1 and AP3 have more pronounced alterations than AP2. In the oxidative stress condition, AP1 is overexpressed, while AP2 shows a different behaviour, being downregulate (Figure 1B, Ox). AP3

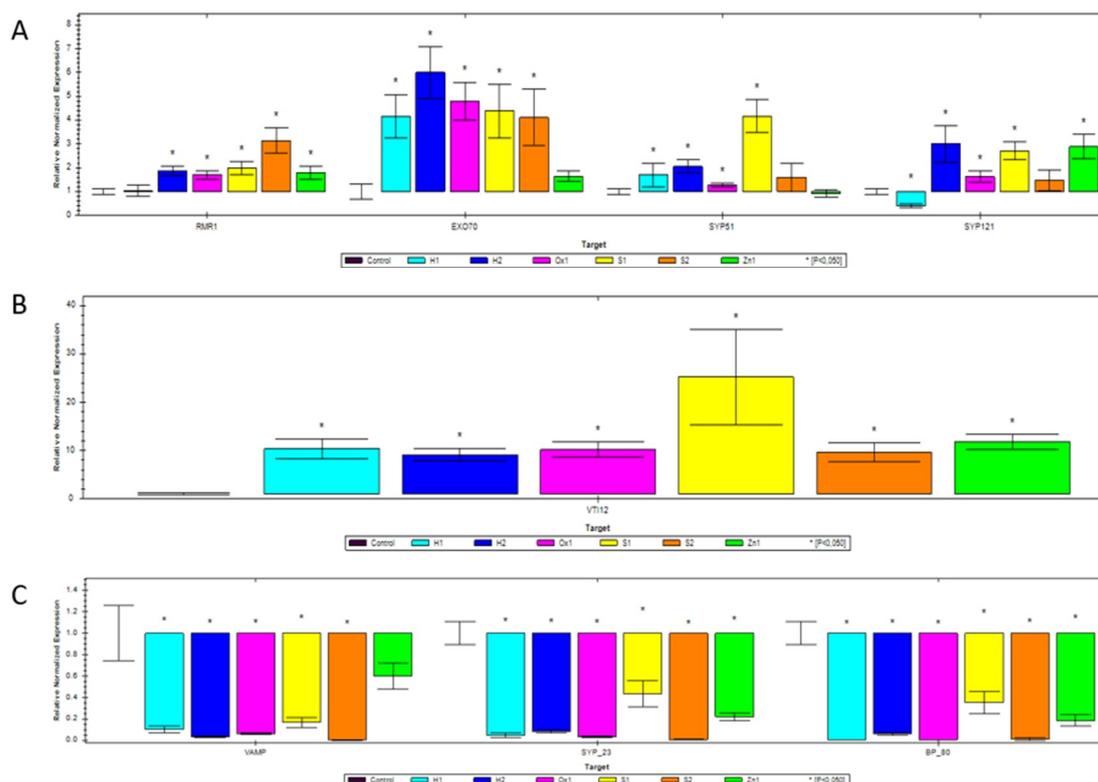
does not show significant changes. For the heavy metal stress, AP1 is upregulated while AP3 is downregulated (Figure 1B, Zn). AP2 does not change its expression significantly. Overall, AP1 responds positively to all stress conditions tested, being its expression ore pronounced in the saline and hydric stress. AP2 is the gene that varies less, having a significant response only in the higher saline condition and in oxidative stress. AP3 expression also changes with the stress, but with a less expression than AP1. In the heavy metal-induced stress AP1 and AP3 have antagonic responses in terms of expression, being the first upregulated and the second downregulated.



**Figure 1.** qRT-PCR results of AP target genes. (A)–Comparison of the 3 AP genes expression in control situation, relative to 0. (B)–Expression of AP genes under stress conditions compared to control situation. \* indicates statistically experimental values ( $p < 0.05$ ).

### 3.2. Expression of Endomembrane System Effectors under Stress Conditions

Several endomembrane system-associated genes were selected for this study based on their role, localisation and putative involvement in unconventional pathways. Their expression was evaluated in the same plants, germinated under stress conditions, as the aspartic proteinases' genes and compared to control conditions. Almost all the genes selected showed alterations in their expression being either upregulated or downregulated (Figure 2A–C). The results obtained allowed to group these genes in two groups: one where all are upregulated (Figure 2A) and a second one, where all the genes are downregulated (Figure 2C). In the first group are present *AtRMR1*, *AtEXO70*, *AtSYP51* and *AtSYP121*. *AtRMR1* is overexpressed in all situations, except in H1, being the maximum observed in S2 samples. *AtEXO70* is overexpressed in all the samples tested, except on the heavy metal stress, where no significative differences were observed. *AtSYP51* is also overexpressed with a maximum peak in the S2 saline condition. *AtSYP121* presents significative overexpression values in almost all the conditions but is downregulated in H1. The second group comprises the genes *AtVAMP*, *AtSYP23* and *AtBP-80*, that show very similar responses in all the conditions tested (Figure 2C). The expression of *AtVTI12*, despite being upregulated for all the condition tested, was not included in any group because the relative expression is much higher, when compared to the other genes (Figure 2B). In most of the stress conditions is roughly 10 times higher than the control (for the other genes it varies between 2 and 6 fold higher) and in the saline S1 condition the expression is 20 to 30 fold higher than the control.



**Figure 2.** qRT-PCR results of endomembrane-related genes. (A)–Expression of *AtRMR1*, *AtEXO70*, *AtSYP51* and *AtSYP121*, relative to control; (B)–Expression of *AtVTI12*, relative to control control situation; (C)–Expression of *AtVAMP723*, *AtSYP23* and *AtBP80*, relative to control \* indicates statistically experimental values ( $p < 0.05$ ).

#### 4. Discussion

During their life cycle, plants can be exposed to several adverse conditions, including salinity, drought, extreme temperatures and heavy metal poisoning, commonly characterized as abiotic stresses. These conditions will affect several molecular, biochemical and physiological processes resulting in delays in development and eventually senescence and cell death [21]. Because plants are not able to move, they cannot respond to environmental stimuli as animals, and adequate responses at the cellular level became particularly important. The literature is rich of data regarding plants, and particularly crops, phenotypes and physiological responses to several types of abiotic stress. Despite several regulators (positive and negative) have been described so far, the regulatory molecular mechanisms entailing the basis of plant adaptation to stress remain essentially unknown.

##### 4.1. The 3 Typical APs from *Arabidopsis thaliana* Are Differentially Expressed under Stress

Across evolution, plants developed mechanisms and responses in order to face, and adapt to, adverse environmental conditions. Plant proteases are one of the most relevant class of proteins with a role in plant resistance to herbivores and pathogens, being recognized as key regulatory agents [22]. Their role in abiotic stress responses is by far less studied, yet some evidences point to a correlation of the expression levels of proteolytic enzymes with abiotic stress. A class of proteases that are believed to play a role in in abiotic stress are aspartic proteinases (APs, EC3.4.23). APs are endopeptidases widely distributed along the plant kingdom and like their animal counterparts, have activity at acidic pH and are specifically inhibited by pepstatin A [23]. Analysis of the *Arabidopsis* genome showed that this plant have 51 genes coding for APs, that were grouped, based on their domain organization, into 3 different groups: typical, atypical and nucellin-like [24]. A distinguish feature of typical APs is the presence of a 100 aminoacid long insertion, termed Plant Specific Insert (PSI), that is involved in vacuolar sorting and able to induce vesicle leakage, suggested to have a role

in plant defense against pathogens [10,25–27]. In this study we focused our attention in the A1 group of typical APs, composed of three different genes: *At1g11910*, *At1g62290* and *At4g04460*. We examined the expression of each gene in seedlings germinated under different abiotic conditions and compared their expression in relation to control situations. It is worth to mention that all three genes revealed similar expression levels in control seedlings, indicating that the changes in expression observed in this study are only related to the experimental conditions. Interestingly, all three AP genes behave differently in response to different types of stress, and in some situations their response is even antagonistic. *At1g11910* expression is enhanced when compared to the other two genes, specially in the saline and hydric stress conditions. A study performed several years ago regarding APs expression in different plant tissues [28] revealed that *At1g11910* mRNA has a more ubiquitous expression than the other two, that have a more restricted pattern: *At1g62290* expression was detected in seeds and *At4g04460* in flowers. This differential expression *in planta* can explain the more remarkable response observed for *At1g11910*, since in this study we obtained the mRNA from seedlings. It can also point to a more important role for this AP in plant cells response to abiotic stress being a good candidate to further studies regarding plant tolerance or adaptation to adverse conditions. Several plant APs have been already implicated in defense or tolerance to abiotic stress associated with water deficit or high salinity. Contour-Ansel and co-workers [29] showed that a typical AP from common bean was highly implicated during water stress being constitutively expressed in drought-tolerant cultivars. Another research paper focusing a grape AP demonstrated that Arabidopsis plants overexpressing that gene showed enhanced tolerance to salt and drought stress, during germination, seedling and mature stage [30]. If in one hand salt and drought stress are reasonably well documented, the role of proteinases in oxidative and heavy metal stress has not been explored till date. The data obtained in our study regarding these two types of stress is quite intriguing if we consider the results obtained for *At1g62290* and *At4g04460*: in the oxidative stress the first is downregulated while the expression of the second remains unaltered and the opposite scenario is observed for the heavy metal stress. At this point it is not clear the significance of these results, since not much information is available regarding either these two APs or the effect of these conditions in proteases expression in general. However, it would be worth to explore in more detail these observations in order to understand how and why this specificity exists and if it could be related with the expression in the plant and/or their intracellular localization or pathways.

#### 4.2. Genes Involved in the Path to the PSV Are Positively Regulated under Stress

Endomembrane trafficking is a essential process for all eukaryotic cells as several proteins are constantly being produced and need to be delivered to the correct location in order to exert their function. The molecular mechanisms and effectors underlying such pathways have been extensively studied over the years and nowadays a very detailed information is available [31]. The endomembrane system in plants needs to be constantly adapting either to morphological demands related to development or to a changing environment. Thus, trafficking pathways and its associated machinery are tightly linked to stress signalling pathways, for the *de novo* expression and/or re-location of stress-related proteins [32]. Here, we show the expression of several endomembrane-related genes involved in different vacuolar pathways during abiotic stress conditions. The 8 genes selected for this work clustered in two groups: one where the genes were upregulated in all the stress situations (*AtRMR1*, *AtEXO70*, *AtSYP51*, *AtSYP121* and *AtVTI12*) and other where the genes were downregulated for all the stress conditions (*AtVAMP723*, *AtSYP23* and *AtBP80*). Remarkably most of the genes that are overexpressed are associated with the route to the protein storage vacuole (PSV). RMR1 is a vacuolar sorting receptor found to localize to the Golgi, TGN and PSVs and transports proteins that carries a C-terminal vacuolar sorting determinant (ctVSD). Is has been implicated in the sorting of storage proteins to the PSV via precursor accumulating vesicles (PACs) [19,33,34]. On the other hand, BP80, another vacuolar sorting receptor, is responsible for protein transport to the lytic vacuole and through a different pathway and mechanism: BP80 recognizes a typical NPIR motif present at the N-terminus of vacuolar proteins and mediates trafficking through chlatrin-coated vesicles (CCVs) [34,35]. In our study, *AtBP80* is clearly downregulated for all the stress conditions

analysed. Taken together the results obtained for these two vacuolar receptors it is likely that the vacuolar transport is biased towards the PSV pathway. This hypothesis is quite exciting as it indicates that plant cells are able to shift their sorting mechanisms towards a more restrained state and probably start to accumulate storage molecules to face the adverse conditions. This hypothesis gains more strength when we look at the other genes that are upregulated in this study. SYP51 and VTI12 belong to different functional classes of SNARE proteins and both showed to be positive regulators of proteins with a CtVSD in their sorting to PSVs, as was demonstrated for chitinase A [36–38]. To our surprise, *AtVTI12* showed a much higher increase in relative expression when compared to other genes tested, specially during salt stress (approx. 30 times higher). This increase must be related to other function of this protein other than mediating protein trafficking to the PSV. It was demonstrated that VTI12 also have a role in the plant autophagy pathway [39], a process that plays a crucial role in plants adaptation to adverse conditions either derived from environmental stress or pathogen attack [40]. Thus it is not surprising that members of this pathway are upregulated in such process. SYP121 is another SNARE that we found to be upregulated under stress conditions. This protein localizes at the plasma membrane has been implicated in stomatal closure and K<sup>+</sup> channel activity following stress signals [41,42]. SYP121 is therefore important in the control of cellular volume and osmotic adjustment which correlates well with the results observed for the salt and drought stress, since the increase in gene expression in these conditions was more obvious than in oxidative or heavy metal stress. The last gene found to be upregulated in this study is *AtEXO70*, a key player in exocyst-positive organelle (EXPO) formation. It has been involved in autophagy processes and in the vacuolar pathway in animals and plants [43]. Similarly to what we observed for abiotic stress, some EXO70 isoforms were found to be upregulated under biotic stress conditions [44]. Similarly to what has been described for VTI12, the role of this protein in the autophagic process and/or in EXPO could be important for the protein degradation and membrane remodeling occurring during stress events. The expression of two other genes was tested under this study: *AtSYP23* and *AtVAMP723*. Both showed to be downregulated in all stress conditions. Despite not being as well characterized as their homologues, the proteins coded by these two genes have curious localizations: most of the SNARE proteins are found at the post-Golgi level, however, VAMP723 is found at the Endoplasmic Reticulum [45] and SYP23 is a cytoplasmic protein, which lacks a transmembrane domain [46]. At this point it is not possible to assess the biological relevance of the observed behaviour of these two genes in abiotic stress conditions, nevertheless it is worth to evaluate the expression of other members of their family in order to understand their role in this process.

## 5. Conclusions

It is known that adverse environmental conditions challenge the endomembrane organization of the cell that need to readjust protein biogenesis and trafficking pathways. In the present study we analysed the expression of several genes involved in the vacuolar pathway during stress conditions. From the results obtained we conclude that the vacuolar pathway to the protein storage vacuole is enhanced, as evidenced by the upregulation of the aspartic proteinases' genes and of vacuolar receptors and members of the SNARE family involved in this route. Also the autophagic pathway seems to be enhanced as well, confirming its role during abiotic stress conditions. In order to sustain the data presented here, it would be interesting to test the expression and localization of other genes involved in the pathways here described.

**Author Contributions:** J.N., A.S., S.P. and C.P. conceived and designed the experiments; J.N. performed the experiments; J.N., A.S. and C.P. analyzed the data; J.P. contributed reagents and materials; J.N. 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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