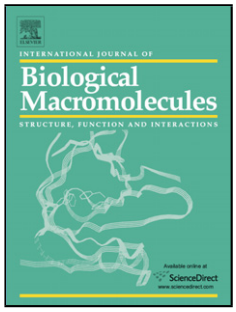

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Role of the durum wheat dehydrin in the function of proteases conferring salinity tolerance in Arabidopsis thaliana transgenic lines

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Abstract

Dehydrins are claimed to stabilize macromolecules against freezing damage, dehydration, ionic or osmotic stresses, thermal stress and re-folding yield. However, their precise function remains unknown. In this context, we report the behavior of protease activities in dehydrin transgenic *Arabidopsis* lines against the wild type plant under salt stress (100 mM NaCl). Indeed, proteases play key roles in plants, maintaining strict protein quality control and degrading specific sets of proteins in response to diverse environmental and developmental stimuli. We proved that durum wheat DHN-5 modulates the activity of some proteases, summarized on the promotion of the Cysteinyl protease and the decrease of the Aspartyl protease activity. This fact is also upgraded in salt stress conditions. We conclude that the dehydrin transgenic context encodes salinity tolerance in transgenic lines through the modulation of the interaction not only at transcriptional level but also at protein level and also with the impact of salt stress as an endogenous and exogenous effector on some biocatalysts like proteases.

Keywords: Salinity tolerance; dehydrin transgenic context; Cysteinyl-protease; Aspartyl-protease; transgenic Arabidopsis lines and biocatalyst modulation.

Introduction

Dehydrins (LEA group 2 family) are plant proteins that are responsive to abiotic stresses such as drought and high salinity [1-3]. Late embryogenesis abundant (LEA) proteins are members of a large group of hydrophilic, glycine-rich proteins found in plants, algae, fungi, and bacteria that are preferentially expressed in response to dehydration or hyperosmotic stress. This group of proteins can be found in seeds at the later maturation stage and/or in the desiccated seed [4-6].

On another hand, plants are equipped with hundreds of genes encoding proteases, with large proteolytic machinery that modulates the fate of proteins [7, 8]. This machinery has generally been viewed in a housekeeping role, like the removal of non-functional proteins and the release of amino acids for recycling in the biosynthesis systems [8]. However, proteases also appear to play key roles in the regulation of biological processes in plants beyond classical protease roles in starvation, stress response and nutrient remobilization [7, 9]. Nevertheless,

these phytozymes play crucial roles in many other important processes in plant cells, e.g. responses to changes in environmental conditions, senescence and cell death [8]. Very little information is available on the substrate specificity and physiological roles of the various plant proteases. In *Arabidopsis thaliana* genome, many genes with sequence similarities to known proteases have been identified; the MEROPS database of Arabidopsis proteases were estimated to almost 3 % of the proteome [8].

Certain Cysteinyl proteases called "caspases" play a key role in animal apoptosis [9]. The involvement of plant proteases in signaling during hypersensitive response has been predicted on the basis of a seemingly conserved 'death pathway'. Although plant caspase genes have not yet been identified, their existence is indicated by many studies in which caspase inhibitors blocked the hypersensitive response and other defense ones. Further evidence that plant proteases are involved in defense emerged recently with the identification of RCR3, a secreted Cysteinyl protease that is required for the function of the resistance gene Cf-2 [10] and CDR1, a secreted Aspartyl protease that regulates defense responses [11].

Thus, as reported here, we undertook the role that can be played by the durum wheat dehydrin (DHN-5) on the acquisition of salinity tolerance in transgenic lines, through the modulation of some enzymatic activities, especially Cysteinyl protease and Aspartyl protease which are up and down regulated in dehydrin transgenic context [12]. We report also the contribution of the transgenic context and salt context both and alone on those phyto-biocatalysts behaviors in order to explain the nature of the plausible function played by DHN-5 in this case and also to give response to the question related to the mode of action so as the mechanism that can be adopted by dehydrin to confer salinity tolerance in the studied transgenic lines.

Materials and methods

Growth and salinity treatments

The two transgenic *Arabidopsis* lines overexpressing durum wheat dehydrin *Dhn-5* gene have been previously described by Brini et al. [4, 5]. Both transgenic *Arabidopsis* lines and the wild-type plants were grown on MS agar medium [13] for one week under light/dark cycle condition of 16 h light/8 h dark cycle at 22°C, and then transferred to MS medium supplemented or not with 100 mM NaCl [4].

Preparation of the enzymatic extract

Aliquots of frozen fresh shoot material (0.5 g) were ground to a fine powder with liquid nitrogen and homogenized in a cold solution containing 100 mM Tris-HCl buffer (pH 8), 10 mM EDTA, 50 mM KCl, 20 mM $MgCl_2$, 0.5 mM PMSF. The homogenate was centrifuged at 14.000 × g for 30 min at 4°C and the supernatant was used for determination of the enzyme activities. Protein concentration was determined according to Bradford method [14].

Protease assay

Protease activity was routinely determined by using Hammerstein casein (Merck, Darmstadt, Germany) as a substrate. Enzyme solution (0.5 ml) suitably diluted was mixed with 0.5 ml 100 mM Tris–HCl buffer at pH 8 containing 1% casein, and incubated for 15 min at 60 °C. The reaction was stopped by the addition of 0.5 ml TCA 20% (w/v). The mixture was allowed to stand at room temperature for 15 min and then centrifuged at 10,000 × *g* for 15 min to remove the precipitate. The acid soluble material was estimated spectrophotometrically at 280 nm. A standard curve was generated using solutions of 0-100 µg/L tyrosine. One unit (U) of protease activity was defined as the amount of enzyme, which yielded 1 µg of tyrosine per minute under the experimental conditions used. Specific activities are expressed as U/mg of protein [15]. The determination of protease activity was monitored with and without specific inhibitors; the inhibitor used in the case of Cysteinyl protease is iodoacetamide at the concentration of 5 mM [16] and the inhibitor used in the case of Aspartyl proteases is pepstatine A at the concentration of 1 µg/ml [16].

Determination of sodium contents

To determine Na^+ content in wild type and dehydrin transgenic lines, leaves were carefully separated from roots, rinsed in deionized water, blotted with filter paper and oven dried at 70 °C until constant mass was reached. Dry material was mineralized using 0.5 N HNO_3. After centrifugation to remove debris, the supernatants were analyzed by atomic absorption spectrometry [17].

Effect of sodium chloride on protease Assay

Protease activities were monitored in the presence of sodium chloride (NaCl at the appropriate concentrations as described by Saibi et al [18, 19].

***In silico* tools**

Each protein network was analyzed to unfold its interactions with other proteins using STRING version 10 database (Search Tool for the Retrieval of Interacting Genes, available at: http://string-db.org/) [20]. We restricted the network to only 10 interactors. The prediction methods selected for our analysis include Neighbourhood, Gene Fusion, Co-occurrence, Co-expression, Experiments, Databases and Text mining.

Statistical analysis

Data were analyzed using one-way analysis of variance and treatment mean separations were performed using Duncan's multiple range tests at the 5 % level of significance.

Results and discussion

Durum wheat dehydrin named DHN-5 was isolated and characterized so as to study and understand their physiological role. Furthermore, DHN-5 overexpression in *E. coli* and in *Arabidopsis thaliana* was carried out [21].

Performance of DHN-5 in vitro

The first heterologous overexpression was used to decorticate their plausible functional capability in vitro [6]. Hence, we proved for the first time that DHN-5 plays a molecular chaperone role by upgrading the thermoactivity, the thermostability and also the refolding yield of some fungizymes (like bglG) [22] and commercial enzymes like LDH, GOD [6, 23]. At the same case, we proved that DHN-5 is an intrinsically unfolded protein having a peculiar multifunctionality such as antifungal and antibacterial effects, antiaggregant capacity and also chelation potentialities [1, 24, 25].

Potentialities of DHN-5 in vivo

The second heterologous overexpression was carried out to investigate their capacity in vivo. We proved that DHN-5 confers the salinity tolerance to the dehydrin transgenic *Arabidopsis* lines (DH2 and DH4) through the modulation of some of metabolic pathways such as proline metabolism and ROS scavenging system [2].

Take advantages of the *Arabidopsis thaliana* genomic data

The analysis of *Arabidopsis thaliana* genomic data indicates, as followed in figure 1-A and 1-B that the genome of our biological matrix is composed of 723 genes encoding proteases distributed in 5 subfamilies as indicated in figure 1-B. Indeed, there are 33 genes from Threonyl protease subfamily (representing 4.56% from the total protease genes number), 188 genes from Cysteinyl protease subfamily (26%), 321 genes encoding Seryl proteases (44.39%), 87 genes encoding Aspartyl proteases (12.03%) and, finally, 94 genes encoding metallo proteases (13%). The analysis of the data given by the affymetrix gene chip microarray ATH1 realized by Brini et al [21], among approximately 24.000 genes, only 77 genes were significantly affected in DH4 compared to the wild type under stress condition (100 mM of NaCl). Within this group, there are ones that encode Cysteinyl and Aspartyl proteases [21]. At this level, we guess to resolve the nature of role played by dehydrin in this case at only transcriptional level or at protein-protein interaction level?

Figure 2 illustrates some properties of those two proteases. Indeed, the first one is a Cysteinyl protease (AT2G27420) that is composed of 348 amino acids having a molecular weight of 37.73 kDa and a computed isoelectric point of 5.10. The second one is defined as an Aspartyl protease (AT3G59080) of 535 amino acids, 59.24 kDa and a computed isoelectric point of 5.33.

Promotion of the total protease activity in dehydrin transgenic lines

The analysis of the curve of figure 3 shows a clear improvement of the total protease activity in transgenic lines (DH2 and DH4) compared to the wild type plant (Wt) under standard conditions. Indeed, the total protease activity was 78.27 U/ml in the wild type plant compared to dehydrin transgenic plants (DH2 and DH4) that were in the range of 107.27 and 106.66 U/ml, respectively. The same variable measured under stress condition was 80.9 U/ml in the wild type plant and 151.27 and 155.18 U/ml in the dehydrin transgenic lines (DH2 and DH4), respectively.

This improvement is accentuated in the presence of salt (100 mM NaCl). In conclusion, we can say that the total protease activity is positively affected in transgenic context. This improvement depends also on the salt stress. Hence, we can note that the enhancement of the total protease activity is followed due to two factors; the first one is the presence of dehydrin and the second one is the stress condition applied during these studies.

The assessment of the Cysteinyl and Aspartyl protease activities

The Cysteinyl protease activity was measured as showed in figure 4-A in the presence of the specific Aspartyl protease inhibitor (pepstatin A at the concentration of 1 µg/ml), we note that this phytozyme is upgraded in dehydrin transgenic context per comparison to the wild type one. Indeed, the activity in wild type was 50.63 U/ml and in DH2 and DH4 were 76.54 and 77.36 U/ml, respectively. In addition, this improvement is enhanced only in dehydrin transgenic lines under stress condition (NaCl, 100 mM) in that the activity is ranged to 87.63 and 95.63 U/ml, respectively. These findings corroborate with the fact that DHN-5 and salinity stress are responsible on this increase in activity. However, the contribution of the dehydrin is judged the main and the basic factor encoding this improvement, compared to the salt stress.

Concerning the Aspartyl protease activity, it was determined at the same experimental condition, in presence of the specific inhibitor of the Cysteinyl protease one (iodoacetamide at the concentration of 5 mM). In this case and as showed in figure 4-B, the Aspartyl protease activity is monitored to 22 U/ml in the wild type plant and 28 and 33 U/ml in the dehydrin transgenic lines (DH2 and DH4), respectively. It was 19, 11 and 9 U/ml under the stress condition, respectively. These facts reinforce the suggestions determined after the analysis of the data followed in figure 4-A.

Effect of the transgenic and the salt stress contexts on the studied protease activities in vivo

Based on the findings given by the analysis of the data in figure 3 and 4, we can deduce the contribution of the dehydrin transgenic context and also the salinity one to clarify the level of implication or the contribution of such factor, taken alone, in the regulation of the protease

activity in this case. We thought to mimic the transgenic and the salt stress contexts as effectors on those phytobiocatalysts assessment in vivo. Indeed, table 1 clearly illustrates the degree of contribution of these two factors, each alone and both combined. Based on the analysis of these results, we can say that the contribution of the transgenic context was determined on the total protease activity to +37.07% and 36.27% in DH2 and DH4, respectively, per comparison to the wild type used as matrix. This effect was determined on the Cysteinyl protease and Aspartyl protease to +51.17% and +27.27% in DH2 and +52.79% and +50% in DH4, respectively.

In addition, the contribution of the both effectors (transgenic and salt stress contexts) was determined on the total protease activity to +93.26 % and +98.26 % in DH2 and DH4, respectively, per comparison of the wild type used as matrix cultivated under standard condition (MS medium). This effect was determined on the Cysteinyl protease and Aspartyl protease to +73.08 % and -50 % in DH2 and +88.88 %, and -59.10 % in DH4, respectively. At the same case, the contribution of the salt stress context on the total protease, Cysteinyl protease and Aspartyl protease activity was monitored to +3.36 %, +4.12 % and -13.64 %, respectively.

Sodium chloride modulates protease behavior in vitro

In order to better understand the impact of the saline context on enzyme activity in vivo, thus the contribution of the sodium on the modulation of the turn-over of the protease activities; we proposed to seek for the effect of this salt (NaCl) in vitro. To do this, the total protease, the Aspartyl protease and the Cysteinyl protease activities were determined. The question that can be asked before is: what is the salt concentration that we have to use so as to monitor the effect of salt on the enzyme activities in vitro to mimic the real in vivo context? For that, we determined the content on sodium in wild type and dehydrin transgenic line (DH2 and DH4) leaves, under standard and stressed condition. The findings of the flame spectrometry illustrated in figure 5 indicate that the sodium effector dose is of the order of 3.2 and 2.5 mM in wild type and in dehydrin transgenic lines, respectively. Furthermore, the relative activity was determined in the presence of salt solution at those concentrations. Indeed, table 2 indicates that the residual total protease activity is of 115% in the wild type and 165% in DH2 and 175% in DH4 lines. The Cysteinyl protease is estimated to 108% in the wild type, 138%

in DH2 and 141%, in DH4 lines. Aspartyl protease activity is of 111% in the wild type, 118% in DH2 and 116% in DH4 lines.

Interactome findings analysis

The study of protein interaction of the two target proteins (Cysteinyl protease and Aspartyl protease), taken alone, with the proteome of Arabidopsis thaliana showed, as followed in figure 6, that each one has ten potential partners, clearly indicated in table 3 and table 4, respectively. Among aspartyl protease partners, we can cite SZF1. Indeed, Jiaqiang Sun and collaborators showed that AtSZF1 is closely related to CCCH-type zinc finger proteins, and it is involved in salt stress responses in Arabidopsis.

In fact, they prove that mutants disrupted in the expression of *AtSZF1* exhibit increased expression of a group of salt stress-responsive genes in response to high salt [26]. Taken together, these results demonstrated that AtSZF1 negatively regulates the expression of salt-responsive genes and plays important roles in modulating the tolerance of Arabidopsis plants to salt stress [26]. We can note that the interaction relationship between the Aspartyl protease and the AtSZF1 reinforces the implication of the studied enzymes on the modulation of the tolerance of plant against salt stress under the dehydrin transgenic context (line DH2 and DH4). It seems that the interaction of Aspartyl protease and AtSZF1 in dehydrin transgenic context may be reinforced giving birth to a new micro-environment. Indeed, we can say that the transgenic context gives to the plant a tolerance to salt stress through its multifactorial interactions, following two types of modulation (positive and negative regulations). This modulation may be dictated by the disordered aspect of the DHN-5 and its multifunctionality, already proven [1, 6, 24, 25].

Conclusion

DHN-5 was described as a multifunctional protein through the crucial roles played such as the thermoactivator and the thermostabilisator roles on some of biocatalysts and also the ability to chelate metals, especially sodium chloride [1, 6]. Added to the role of DHN-5 already described during the modulation of proline metabolism and the ROS scavenging system related to the acquisition of salinity tolerance in transgenic lines [2], we can add according to

findings presented here the protein-protein interaction which confers tolerance to salt stress. At this state, we note that these interactomic analyses are based only on in silico-obtained results and they need to be validated by interactomic experiments such as yeast-two-hybrid, split-GFP, or other approach. The dehydrin seems to be a key protein that plays a heat protective role [6] and interacts with multiple partners involved in different pathways, such as proline metabolism [2]. All these findings reinforce the importance of this protein in plant stress response.

Acknowledgments

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Figure Captions

Figure 1: Repartition of the *Arabidopsis thaliana* proteases.

Figure 2: Illustration of the studied Cysteinyl protease and Aspartyl protease properties in *Arabidopsis thaliana* genome.

Figure 3: Histogram illustrating the total protease activity in the wild type and in dehydrin transgenic lines (DH2 and DH4). Values are mean ± SE (n = 5). Asterisks indicate significantly greater mean values compared to Arabidopsis control line ($P < 0.05$).

Figure 4: Plots showing the Aspartyl and Cysteinyl protease activity in standard and stress (NaCl 100 mM) conditions. Values are mean ± SE (n = 4). Asterisks indicate significantly greater mean values compared to Arabidopsis control line ($P < 0.05$).

Figure 5: Illustration of sodium content in leaves using flame spectrometry. Values are mean ± SE (n = 4). Asterisks indicate significantly greater mean values compared to Arabidopsis control line ($P < 0.05$).

Figure 6: Plots illustrating the interaction of the two target proteins with the *Arabidopsis thaliana* proteome. **A** arises the interactome of Cysteinyl protease and **B** the Aspartyl one. Aspartyl protease and cystenyl protease are represented with a red sphere**.**

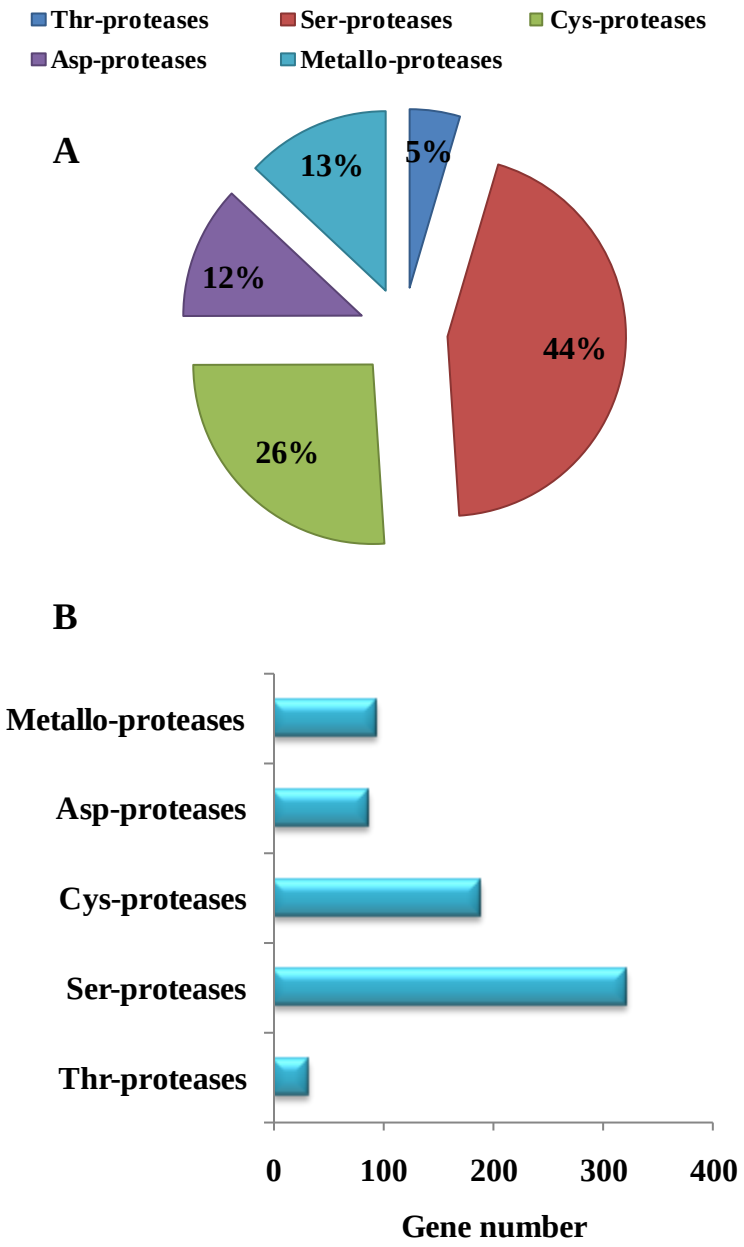

Figure 1

	Locus	Isoelectric point	MW (kDa)	Amino acid number	NT sequence
Cystenyl-protease	AT2G27420	5.10	38.73	348 aa	1047 bp
Aspartyly-protease	AT3G59080	5.33	59.24	535 aa	2042 bp

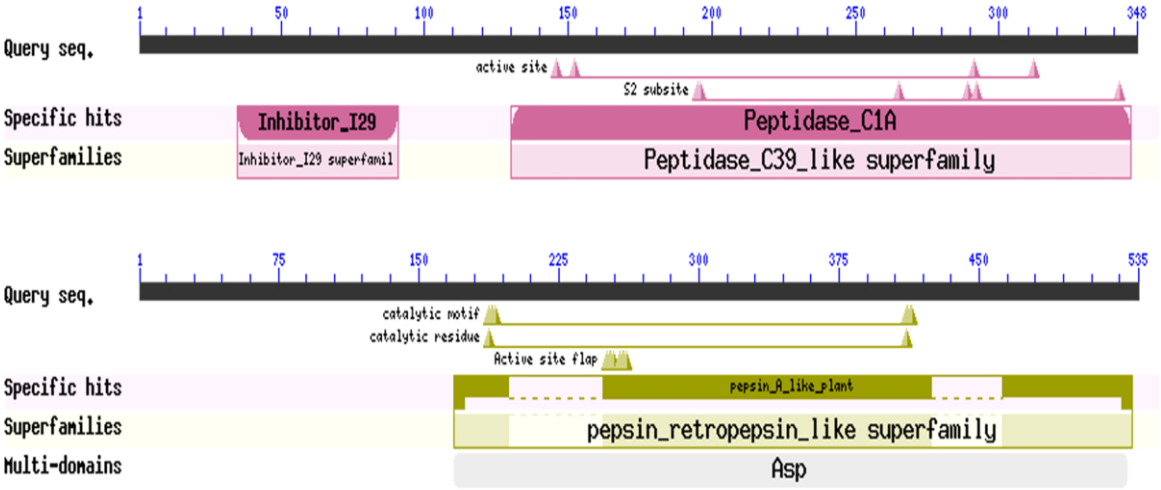

Figure 2

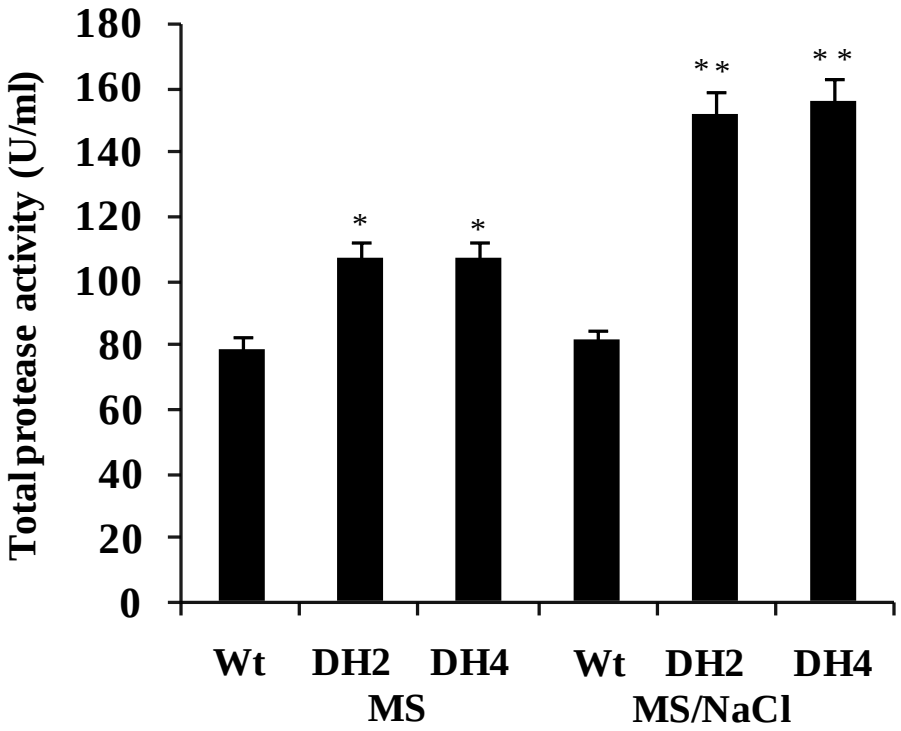

Figure 3

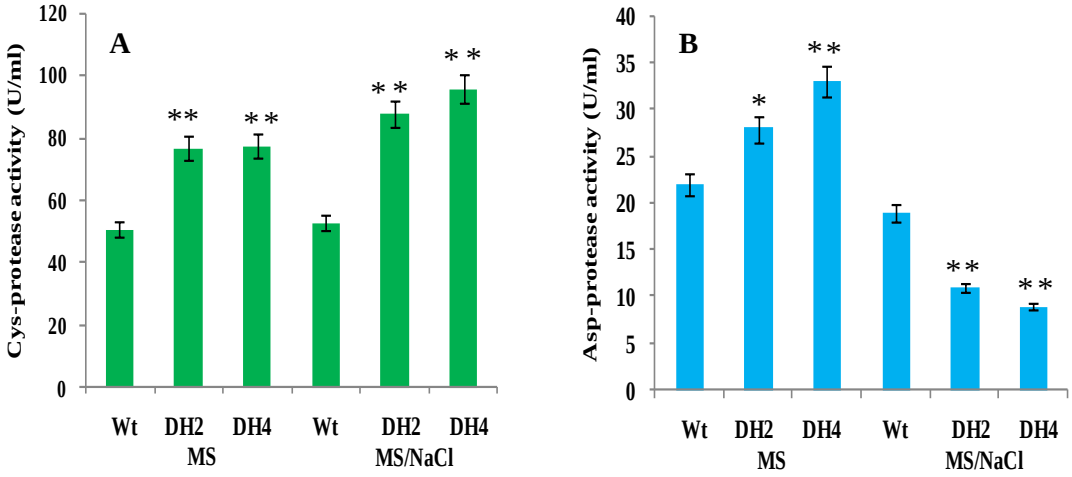

Figure 4

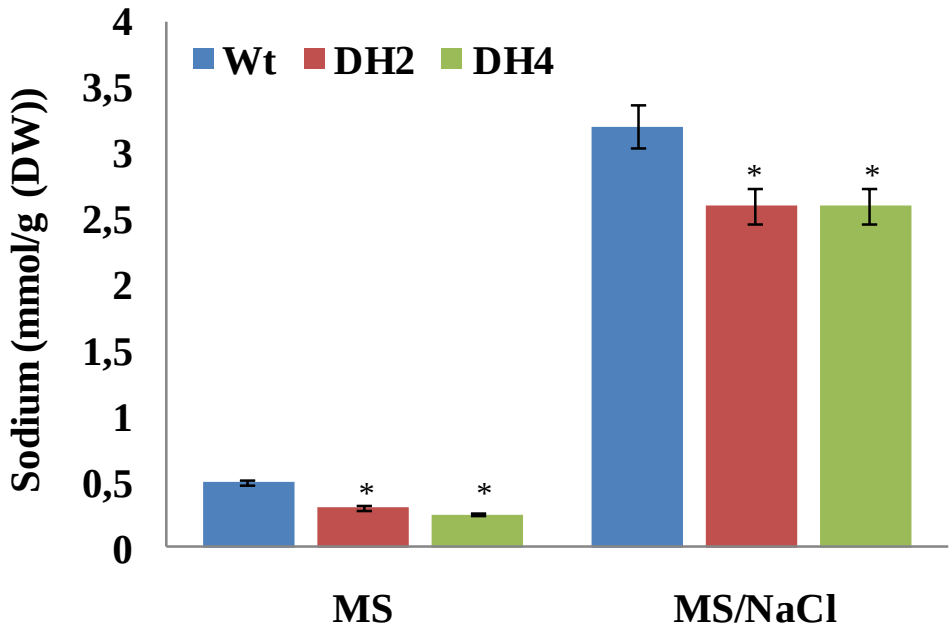

Figure 5

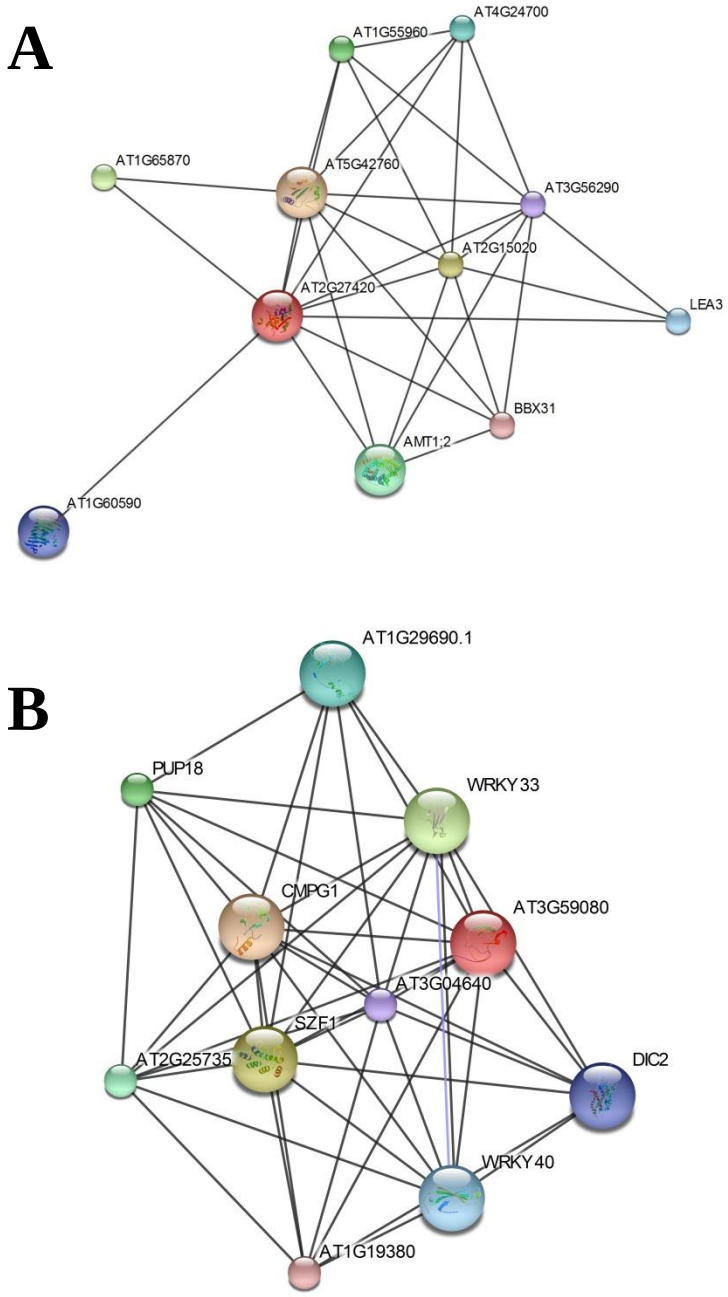

Figure 6

Table 1: Contribution level of the transgenic and salinity contexts on the tolerance to salt stress in transgenic lines (DH2 and DH4)

Contribution level on the salinity tolerance			
Transgenic context contribution			
	Total protease activity	**Cys-protease activity**	**Asp-protease activity**
Wt	**100**	**100**	**100**
DH2	**+37.07**	**+51.17**	**+27.27**
DH4	**+36.27**	**+52.79**	**+50**
Transgenic context and salt stress contributions			
	Total protease activity	**Cys-protease activity**	**Asp-protease activity**
Wt	**+3.36**	**+4.12**	**-13.64**
DH2	**+93.26**	**+73.08**	**-50**
DH4	**+98.26**	**+88.88**	**-59.10**
Salt stress contribution			
	Total protease activity	**Cys-protease activity**	**Asp-protease activity**
Wt	**+3.36**	**+4.12**	**-13.64**

Table 2: Illustration of the effect of sodium chloride on the enzyme activities in vitro

Effect of sodium chloride on the enzyme activities in vitro			
Residual protease activity (%)			
	Total protease activity	**Cys-protease activity**	**Asp-protease activity**
Wt (MS), [NaCl]=0 mM	**100**	**100**	**100**
Wt (MS), [NaCl]=3,2 mM	**115**	**108**	**111**
DH2 (MS) [NaCl]=2,5 mM	**165**	**138**	**118**
DH4 (MS) [NaCl]=2,5 mM	**175**	**141**	**116**

Table 3: Recapitulation of the first ten partners interacting with the Cysteinyl protease in *Arabidopsis thaliana* genome

Protein ID	**Protein role function**	**Score**
AT5G42760	Leucine carboxyl methyltransferase **(364 aa)**	0.779
AT2G15020	uncharacterized protein **(526 aa)**	0.742
AT1G65870	Disease resistance-responsive (dirigent-like protein) family protein **(189 aa)**	0.604
AT1G55960	putative polyketide cyclase/dehydrase and lipid transport-like protein **(403 aa)**	0.559
AT1G64780	AMT1;2:ammonium transporter 1;2; Ammonium transporter probably involved in ammonium uptake from the soil **(514 aa)**	0.510
AT4G24700	uncharacterized protein **(143 aa)**	0.497
AT1G02820	LEA3:late embryogenesis abundant 3 **(91 aa)**	0.463
AT1G60590	Pectin lyase-like protein **(540 aa)**	0.452
AT3G56290	uncharacterized protein **(173 aa)**	0.433
AT3G21890	BBX31:B-box domain protein 31 **(121 aa)**	0.432

Table 4: Recapitulation of the first ten partners interacting with the Aspartyl protease in *Arabidopsis thaliana* genome

Protein ID	**Protein role function**	**Score**
AT1G66160	CMPG1 : CYS, MET, PRO, and GLY protein 1; Functions as an E3 ubiquitin ligase (By similarity) **(431 aa)**	0.904
AT3G55980	salt-inducible zinc finger 1; Involved in salt stress response. May positively modulate plant tolerance to salt stress.**(580 aa)**	0.900
AT2G38470	WRKY33: WRKY DNA-binding protein 33; Transcription factor. Interacts specifically with the W box (5'-(T)TGAC[CT]-3'), a frequently occurring elicitor- responsive cis-acting element (By similarity) **(519 aa)**	0.888
AT1G57990	purine permease 18 **(390 aa)**	0.882
AT2G25735	uncharacterized protein **(119 aa)**	0.878
AT1G29690	constitutively activated cell death 1; Negatively controls the salicylic acid (SA)-mediated pathway of programmed cell death in plant immunity **(561 aa)**	0.846
AT1G80840	WRKY40:WRKY DNA-binding protein 40; Transcription factor. Interacts specifically with the W box (5'-(T)TGAC[CT]-3'), a frequently occurring elicitor- responsive cis-acting element (By similarity) **(302 aa)**	0.832
AT4G24570	DIC2:dicarboxylate carrier 2; PUMPS are mitochondrial transporter proteins that create proton leaks across the inner mitochondrial membrane **(313 aa)**	0.827
AT3G04640	glycine-rich protein **(159 aa)**	0.816
AT1G19380	uncharacterized protein **(147 aa)**	0.808