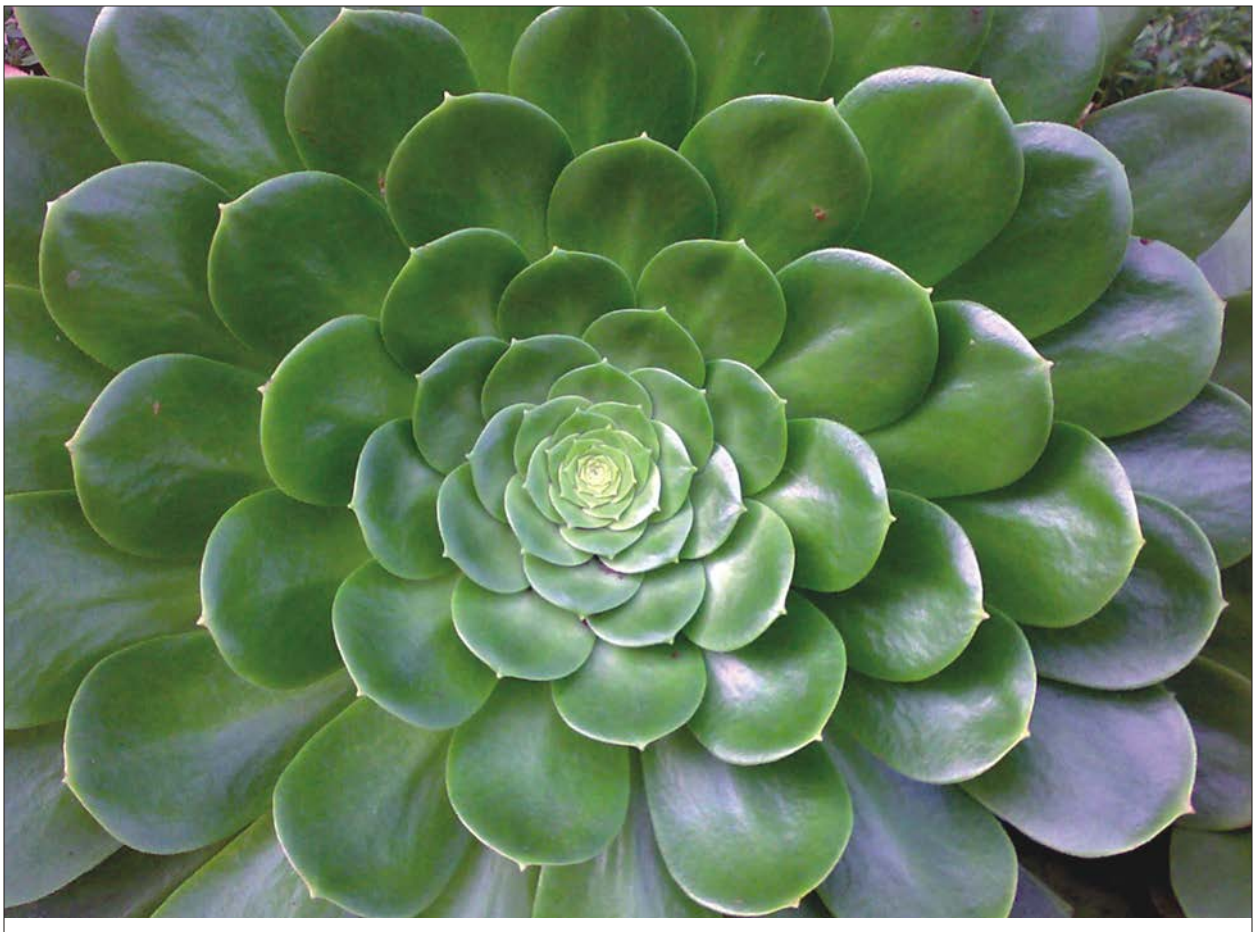

Journal of
Plant Sciences

ISSN 1816-4951

Academic
Journals Inc.

www.academicjournals.com

OPEN ACCESS Journal of Plant Sciences

ISSN 1816-4951
DOI: 10.3923/jps.2022.88.94

Review Article
Biology Adaptation of *Medicago* Legumes for Tolerance Against Salinity Stress Compared to Other Plants

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Abstract

Legumes are very important plants both ecologically and agriculturally for biological nitrogen fixation and soil fertilization. Legumes are also appreciated for their nutritional value for sustainability enhancement. Among these legumes, we have the annual species of *Medicago* and especially the plant model legume *Medicago truncatula* Gaertner. This plant is very rich in protein with high nutritional value and medicinal interest. On the other hand, *Medicago* species show different adaptive responses to environmental conditions like climatic variation and abiotic stress. Salinity represents today, the major abiotic stress that causes land degradation and crop productivity limitation around the world and affects physiology and metabolism in legumes. This present review describes the biology adaptation of the *Medicago* genus to salt stress to understand molecular, physiological and biochemical mechanisms involved in salt stress tolerance.

Key words: *Medicago truncatula* Gaertner, salinity stress, molecular physiology, biochemical mechanisms

Citation: Amouri, A.A., 2022. Biology adaptation of *Medicago* legumes for tolerance against salinity stress compared to other plants. J. Plant Sci., 17: 88-94.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The legume family is large and very diverse and is the most important crop that grows on the surface of the earth, after cereals[1] they live in symbiosis with *Rhizobium meliloti* bacteria and fix atmospheric nitrogen for natural soil fertilization. Among *Medicago* annuals species, *Medicago truncatula* Gaertner is widely used as a model legume plant for understanding tolerance to abiotic stress[2]. This kind of legume is of great interest for sustainable agriculture and ecology and has been used for genetical and physiological studies of root development in legumes. These legumes are rich in protein with nutritional and medicinal interests. Salinity represents today, the major abiotic stress that causes land degradation and crop productivity limitation around the world and affects the crop physiology and metabolism of legumes. Root and shoot length provides important indications of a plant's response to salt stress[3]. Also, the understanding of the relationship between seedling development, environmental conditions and seed quality at the physiological and agronomical levels are basic aims of seed science[4]. This present mini-review describes firstly, how to apply gametophytic selection, a fast and economical technique in *Medicago*. On the other hand, the physiological and molecular approach of proteins and antioxidant metabolism in the root of *Medicago truncatula* to salinity adaptation was addressed.

Male gametophytic selection for salt stress tolerance in *Medicago*: Plant breeding programs are continually being developed in many legumes species to increase agronomic productivity. It would be advantageous to use strategies that allow a rapid selection of tolerant genotypes to salt stress. The male gametophytic selection could be an alternative for legumes breeding. This method is based on the existence of a functional and structural overlapping of gene expression between the responses of pollen (n) and those of the sporophyte (2n) to abiotic stress like salinity.

A functional overlapping of seeds and pollen grain germinations capacities for salt stress tolerance has been demonstrated in annual species of *Medicago* at the haploid (n) and diploid (2n) stage[5]. Figure 1 shows different aspects of pollen germination in four ecotypes of different annuals *Medicago* species like *Medicago truncatula* in Fig. 1a and d and *Medicago ciliaris* in Fig. 1b and c. The effect of different NaCl concentrations on pollen germination *in the Medicago ciliaris* ecotype is illustrated in Fig. 1e-h which shows the decrease of pollen germination ability with increased salt stress degree. In the carrot, it has been demonstrated that pollen viability was also affected by salt stress[6].

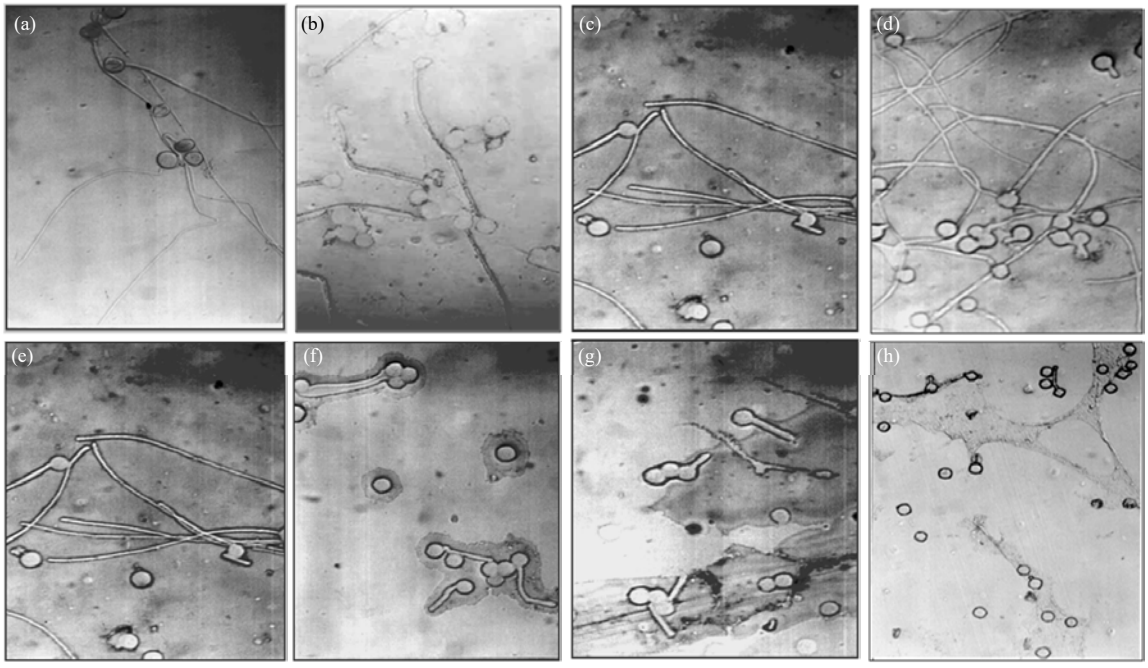

Fig. 1(a-h): Pollen germination in four annual *Medicago* species under normal condition, (a) Tru 42, (b) Cil 242, (c) Cil 56, (d) Tru 242, (e) 0 mM NaCl, (f) 68 mM NaCl, (g) 102 mM and (h) 137 mM[5]
Germination under salt stress conditions in the Cil 56 ecotype

In *Arabidopsis*, it has been shown that changes in protein profiles are associated with the process of pollen development and stress response. In addition, the proteomic study of pollen showed considerable overlap in the presence of proteins simultaneously in mature pollen and seeds of the sporophyte. Notably, both proteomes contain high levels of LEA proteins and chaperones, which have an important role in stress tolerance concerning seed and pollen grain dormancy[7].

Root response to salinity in *Medicago truncatula* Gaertn

Morph-physiological variations: Legumes are very important plants both ecologically and agriculturally because they can interact symbiotically with rhizobia for biological nitrogen fixation and soil fertilization. Recently, *Medicago truncatula* has been chosen as the best legume model because of its small, diploid genome, self-fertility and short life cycle[2] and it's appropriate for discovering new candidate's genes involved in legume breeding to abiotic stress. *Medicago truncatula* is a quite drought tolerant plant species[8]. Salinity stress represents today, the major cause of crop productivity limitation and affects physiology in legumes. The influence can occur in two phases: The first phase is governed by the osmotic effect due to high salt concentration in root zones, whereas the second phase is governed by toxic effects due to high salt accumulation in leaf tissues[9].

The roots are the essential part of the plant that can transmit rapidly a signal stress after perception to other plant parts[4]. The assessment of the variability between eleven ecotypes of *M. truncatula* under salt stress (137 mM of NaCl), showed that the tolerant ecotype Tru 131 had the best root growth compared to the sensitive ecotypes like Jemalong[10,11]. The capacity of plants to tolerate salt stress varies with the phase of growth in their life cycle and seedling emergence is critical for the establishment of plant populations[12]. Zahaf *et al.*[13] examined the root of two contrasting genotypes of *M. truncatula* to salt stress and observed that the tolerant genotype has a good root vigour compared to the sensitive one. The root is the principal part of the plant involved in salinity perception and frequently limits the productivity of plant[14]. Seedling root development is essential for plantlet survival under salinity stress because root growth determines water absorption aptitude[15]. The water potential of the plants becomes progressively more negative with increasing salinity and pressure turgor as demonstrated[16]. The analysis of physiological parameters of roots in two contrasting ecotypes of *Medicago truncatula* to salinity stress showed that the tolerant ecotype decreases water uptake more than the sensitive one and gives good protection against this abiotic stress[17]. The understanding of the relationship between seedling development, environmental conditions and seed quality at the physiological and agronomical levels are basic aims of seed science[4].

Metabolism and biochemical process: In general, high salinity causes oxidative stress affecting plant physiology[18], generating accumulation of "ROS" (reactive oxygen species)[19]. One of the stress defence mechanisms is the antioxidative system, which involves several antioxidant enzymes and non-enzymatic antioxidants such as ascorbate and glutathione[20]. The salt tolerance of alfalfa is also improved by melatonin application, which acts as an antioxidant in scavenging H_2O_2 and enhances antioxidant enzymes' activities[21]. Under salt stress, tolerant alfalfa shows less severe cell membrane damage and lower accumulation of reactive oxygen species (ROS) than salt-sensitive types[22]. Ascorbate (ASC) is the most abundant studied antioxidant compound during environmental stress in plants[23]. Additionally, glutathione (GSH) is the key component of the antioxidant network that scavenges ROS in plant[24]. The ROS also controls the expression of genes in many processes like signalling pathways and plant development[25].

High salinity stress promotes ABA accumulation that induces changes in gene expression[26]. Recently, Wang *et al.*[27] reported that the total ROS content in rice root was higher under salt stress compared with the control.

In *Medicago truncatula* genotypes under salt stress conditions, it was observed a correlation between salinity tolerance and cellular damage[28]. It's was reported that Guaiacol peroxidase is also a key of ROS scavenger[29], this enzyme can be very important for antioxidative defence in the root of *M. truncatula* under salt stress, because plays a vital role in the biosynthesis of lignin as well as defend against stress by degrading indole acetic acid and utilizing H_2O_2 in the process[23]. Zahaf *et al.*[13] expose that the Guaiacol peroxidase activity in *M. truncatula* significantly increases in root nodules in response to salinity in tolerant genotype TN.11 compared to Jemalong A17 a sensitive one. In another study, Pyngrope *et al.*[30] showed that the activity of Guaiacol peroxidase increased in both sensitive and tolerant rice seedlings under water deficit and the extent of increase was greater in the tolerant seedlings than the sensitive. Thus, the study of antioxidants enzymes in relationship with abiotic stress as salinity can be useful in breeding tolerant genotypes. Moreover, NaCl increased guaiacol peroxidase activity in rootlets of the tolerant genotype Tru 131, this enzyme has a protective role against the molecules ROS accumulated during

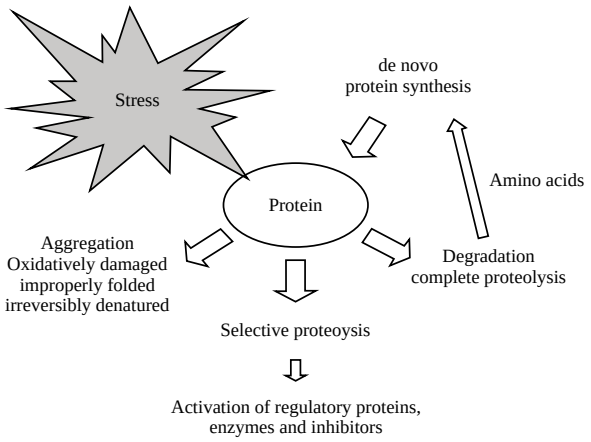

Fig. 2: Role of proteases and proteases inhibitors (cystatins) in plants, under abiotic stress[42]

oxidative stress. On the other hand, under salinity stress, the total content of ascorbate and Glutathione was increased in the tolerant genotype Tru 131 compared to Jemalong. These results show how the tolerant genotype activates the antioxidative defence system at the root level against damages caused by oxidative stress under salinity[17]. However, Agastian *et al.*[31] reported that the soluble protein content is high in low salinity but decreased significantly in the high concentration of NaCl in cultivars of mulberry trees. On the other hand, Das and Roychoudhury[23] reported that the ROS produced during stress conditions causes the oxidation of proteins and chemical modifications such as carboxylation. Protein carbonylation is often used as an indicator for evaluating protein oxidation and degradation[32]. Indeed, the increase in protein carbonyl was used as a marker of oxidative damage in plants exposed to salt stress[33]. In addition, Pyngrope *et al.*[30] showed when rice seedlings of two cultivars were exposed to a water deficit.

Involvement of phytocystatins in plant salt stress tolerance: Proteolysis is an important process for plant growth and seed germination. Among the many types of proteases identified in plants are the cysteine proteases and their inhibitors (Photo Statins). Phytocystatins (PhyCys) are plant proteins that inhibit cysteine proteinases and show variable expression models during plant growth under abiotic stresses[34] and are a class of proteins mainly involved in cysteine protease inhibition and plant growth and development, as well as tolerance under various abiotic stresses. The plant cystatins, homologous to animal cysteine protease inhibitors, have been characterized in several monocotyledons and dicotyledons. Several types of research focused on enzymes like proteinases that are implicated in many cellular reactions involving protein degradation, such as the degradation of storage proteins, their action can be inhibited by cysteine proteinase inhibitors or cystatins superfamily. Some biotechnological advances to plant breeding using phytocystatins, particularly in overexpression studies, have been successful in recent years. Cysteine proteinases play an essential role in plant growth but also, in the accumulation of seed storage proteins and the response to biotic and abiotic stresses[35] and may act as stabilizing fusion partners for recombinant protein production in plants[36].

Several studies suggest that plant cystatins are responsive to abiotic stresses such as drought, salt, abscisic acid and cold treatment[37], although they have also been detected in vegetative tissues, including roots and leaves[38] and play a regulatory role in photosynthesis and respiration[39]. Cysteine proteinases play an essential role in plant growth but also, in the accumulation of seed storage proteins and the response to biotic and abiotic stresses[35]. Their action can be inhibited by proteinase inhibitors induced by abiotic stress. In *Arabidopsis thaliana*, two cysteine proteinase inhibitors (cystatins) designated AtCYSa and AtCYSb, were characterized. The northern blot technique showed that the cystatins gene expressions in seedlings were highly induced by abiotic stresses[40]. The cystatins contain four conserved cysteine residues forming two disulfide bonds and most of them are not glycosylated[41]. Plant cysteine proteinases are localized in vacuoles and the cell wall, they are implicated in the regulation of proteolysis and play a major role in plant responses to abiotic stress and are essential for the maintenance of protein homeostasis[42] in Fig. 2. On the other hand, plant response to biotic stresses, a defence mechanism in plants against insects is determined in maize genotypes by induction of a cysteine proteinase without the involvement of a cysteine proteinase inhibitor[43].

Genetic control of cysteine proteinase inhibitor in *Medicago truncatula*: Cysteine proteinase inhibitor (cystatin) is one of the most important molecules involved in plant development and abiotic stress responses. Expression of the cystatins genes is usually limited to specific organs or particular phases during plant growth under salt stress[39]. The cystatin gene in the grapevine was also induced under abiotic stress[44]. Is still unclear in *Medicago* what are the genes involved in the expression of cysteine proteinase inhibitors enhance salinity tolerance. However, it's interesting to identify and evaluate the regulatory role of these genes. It has been demonstrated that the expressed sequence tag EST-SSR marker (MTIC124) showed that this locus encodes a cystatin "cysteine proteinase inhibitor" and was expressed principally in the root of *Medicago truncatula*[45]. In *Medicago truncatula* anti-sense inhibition of the cysteine protease CYP15A caused a delay

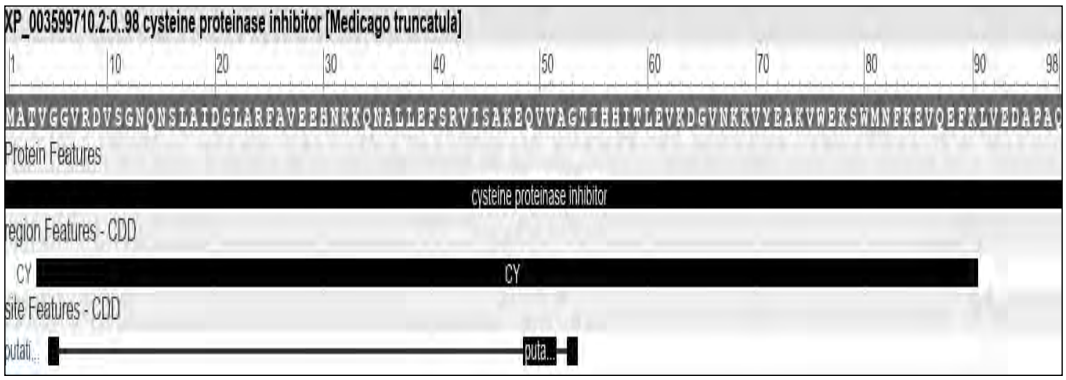

Fig. 3: Graphic representation of cysteine proteinase inhibitor (XP_003599710.2) sequence in *Medicago truncatula*, for salt stress

in nodule senescence[46]. Another salt-responsive gene, *BvM14-cystatin*, was identified from sugar beet and over expressed in *Arabidopsis* with salt tolerance enhanced[47] and also in alfalfa[48].

The putative protein and gene sequences encoded cysteine proteinase inhibitor (cystatin) of *Medicago truncatula* implicated in salt stress tolerance in Fig. 3 were available in GenBank (https://www.ncbi.nlm.nih.gov/protein/XP_003599710.1).

The sequence of this enzyme via the NCBI database is as follow:

- >XP_003599710.2 cysteine proteinase inhibitor [*Medicago truncatula*]
- MATVGGVRDVSGNQNSLAIDGLARFAVEEHNKKQNALLEF SRVISAKEQVVAGTIHHITLEVKDGVNKKVYEAKVWEKSWM NFKEVQEFKLVEDAPAQ

The gene LOC11407914 coding cysteine proteinase inhibitor is located in exon 3 of chromosome 3 of the genome of *Medicago truncatula*.

The sequence of this gene via NCBI data base is as follow:

- >lcl|XM_003599662.3_cds_XP_003599710.2_1[gene = LOC11407914 MTR_3g043750] [db_xref = Gene ID:11407914] [protein = cysteine proteinase inhibitor] [protein_id = XP_003599710.2] [location=71..367] [gbkey = CDS]
- ATGGCAACGGTTGGTGGAGTTCGCGATGTTTCAGGAAAC CAGAACAGTCTTGCGATCGATGGTCTCGCTCGCTTTGCTG TTGAAGAACACAACAAAAAACAGAATGCACTTCTGGAAT TTTCAAGGGTGATAAGTGCTAAAGAGCAAGTGGTTGCTG GTACCATACACCACATCACTTTAGAGGTAAAAGATGGGG TGAATAAAAAGGTTTATGAAGCCAAGGTTTGGGAAAAGT CTTGGATGAACTTCAAGGAGGTTCAAGAGTTCAAGCTCGT CGAAGATGCGCCTGCGCAGTAA

The molecular function of cysteine proteinase inhibitor obtained via QuickGO annotation (https://www.ebi.ac.uk/QuickGO/annotations), showed that it's classified in cysteine-type endopeptidase inhibitor activity family. The role of this endopeptidase inhibitor binds to and stops, prevents, or reduces the activity of a calcium-dependent cysteine-type endopeptidase, any enzyme that hydrolyzes peptide bonds in polypeptides by a mechanism in which the sulfhydryl group of a cysteine residue at the active centre acts as a nucleophile in a calcium-dependent manner. (https://www.ebi.ac.uk/QuickGO/term/GO:0010859). This data suggest the important role of this kind of protease in stress tolerance in plant.

Information is still limited about the regulation of this kind of inhibitors with unclear processes especially in the leguminous *Medicago truncatula* and their possible interaction with proteinases under salt stress conditions, especially at the root level. Interestingly, epigenetic modification as DNA methylation, chromatin remodelling and histone modification can play an important role in regulating gene expression for abiotic stress tolerance and it's important to not neglect this epigenetic aspect.

CONCLUSION

In this mini-review, several studies on abiotic stress showed the role of morphological, physiological and molecular mechanisms involved in salt stress tolerance, principally. The first part of this chapter proved the importance of gametophytic selection in the legume as *Medicago* species for salinity tolerance, the fast and economic technique in plant breeding based on the existence of functional overlapping. The second part focalised on the root response to salinity in the plant model in *Medicago truncatula* Gaertn. This sensitive organ showed various morphological and physiological modifications like root growth and root water potential. Also,

variations were observed at the biochemical level by ROS scavenger, antioxidants and cystatins accumulations in the root. On the other hand, molecular and the in silico approach showed that the genetic control of cystatin "Cysteine proteinase inhibitor" can significantly enhance salts stress tolerance in plants and especially in the model legume *Medicago truncatula* Gaertn. All these scientific investigations can help researchers and breeders with effective plant breeding.

SIGNIFICANCE STATEMENT

This mini-review can be useful for researchers working on plant physiology adaptation to salinity stress in legumes like *Medicago truncatula* that this genome was completely sequenced and more investigations are necessary to exploit and understand molecular physiology adaptation mechanisms linked to abiotic stress.

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