Review Article

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Ekin
Journal of Crop Breeding and Genetics

7(2):106-115, 2021

Ekin International biannual peer-reviewed journal

Transgenics for Improving Salt Stress Tolerance in Legume Crops Chickpea and Pigeon Pea

Pushpa KHARB* **Rinku CHAUDHARY** **Nita LAKRA** **Rishi Kumar BEHL**

Department of Molecular Biology, Biotechnology & Bioinformatics, Chaudhary Charan Singh Haryana Agricultural University, Hisar (Haryana), India

* Corresponding author e-mail: pkharbhau@gmail.com

Citation:
Kharb P., Chaudhary R., Lakra N., Behl RK., 2021. Transgenics for Improving Salt Stress Tolerance in Legume Crops Chickpea and Pigeon Pea. Ekin J. 7(2):106-115.

Received: 28.03.2021 ***Accepted: 31.05.2021*** ***Published Online: 29.07.2021*** ***Printed: 30.07.2021***

ABSTRACT

Legumes, being rich in proteins, are an important part of human diet and account for about 27 per cent of crop production around the globe. Chickpea and Pigeon pea are the two most important legume crops of India. However, production in legume crops is adversely affected due to salinity in arid and semi-arid regions of world. Salt stress reduces water potential, creates imbalance in ion concentration and causes toxicity. Helicases (RuvB & p68) have been shown to play an important role in plants against salt stress. p68 which is a DEAD-box helicase interacts with Ca^{2+} -CaM. LecRLK is another important gene involved in regulating diverse signalling pathways under salt stress condition in plants. The present review highlights the role of salinity stress tolerance by these helicases and lectin receptor kinases genes by developing transgenic chickpea and pigeon pea lines.

Keywords: Chickpea, helicases, LecRLK, legume, pigeon pea, salinity, transgenics

Introduction

Abiotic stress is the major cause of decreasing the yield of important food crops by more than 50%, leading to the losses worth of million dollars every year (Rasool et al. 2013; Lamaoui et al. 2018). Among abiotic stresses, high salinity stress is the most severe environmental stress, which impairs crop production on at least 20% of irrigated land worldwide. Out of the 1500 million hectares agricultural land, 32 million (2%) is affected by secondary salinity of varying degrees. Further, problems will be worsened as nearly 50% of the arable land will hit salinity by 2050 (Machado and Serralheiro, 2017). Extensive economic losses due to salinity include costs of $27 billion-plus loss of crop value per year (Kumar et al. 2017).

Excess of salt in soil is one of the major devastating abiotic stresses for global agriculture as it may cause degradation of arable soils, particularly those that are heavily irrigated via adverse impacts on seed germination, plant growth and development, plant vigour and crop yields and hence drastically reducing agricultural productivity (Cheeseman 2015; Akram et al. 2017; Kumar et al. 2017). A saline soil is defined as one in which the electrical conductivity (EC) of the saturation extract (EC_e) in the root zone exceeds 4 dS/m (approximately 40 mM NaCl) at 25°C and has an exchangeable sodium of 15%. The yield of most crop plants is reduced at this EC_e, though many crops exhibit yield reduction at lower EC_{es} (Munns, 2005; Jamil et al. 2011). The repercussions of salinity stress on crop productivity and concerns regarding its management have been the focus of several prior comprehensive reviews (Hoffman et al. 2007; Grattan et al. 2011; Pereira et al. 2014). The direct effects of excess of soluble salts in soil causes imbalance or accumulation of specific ions (Cl, Na) in plants which results in osmotic stress because of reduced soil water availability and ion imbalance and ion toxicity (Munns 2005) which lead to plant demise ultimately (Maas and Hoffman 1977; Zorb et al. 2014).

Recent estimates show an increase in global salt-affected area with an area of 1,128 million ha

(Mandal et al. 2018). According to Shrivasata and Kumar (2015) approx. 20% of total cultivated and 33% of irrigated agricultural lands worldwide are affected by high salinity. Moreover, the salt affected areas are increasing at a rate of 10% annually for several reasons, including high surface evaporation, low precipitation, irrigation with saline water, weathering of native rocks, and poor agricultural practices. In India, nearly 5% of the net cultivated area is having salt affected soils, spreading from Jammu & Kashmir (Ladakh region) in north to Kanyakumari in south, and Andaman & Nicobar Islands in the east to Gujarat in the west. Increasing trend in the salt-affected soils is becoming a major threat to economic development and national food security in India.

Salinity stress affects plant health in two ways, first is decrease in soil porosity that leads to decrease in oxygen and water movement around the roots and secondly increased Na^+ concentration is toxic for essential enzymes which in turn alter the physiology of plants (Munns and Tester, 2008). It has been reported that when salinity rises to 100 mM NaCl in a field, most of the legume species die before maturity (Munns et al. 2002).

Legumes belong to the important plant family Leguminosae or Fabaceae and provide the prime single source of vegetable protein in human diets and livestock feed (Dita et al. 2006). Legumes can serve as resource-conserving alternative as these plants can fix atmospheric nitrogen, thus plummeting the requirement for chemical fertilizers and hence playing a role in improving soil health and increasing overall crop productivity. Reduction in pests, diseases and weed populations has been observed in farming systems, when legumes are used as an inter-crop. Legumes occupy 12-15% of worldwide arable land to provide 33% of dietary protein and 27% of major crop production (Flexas et al. 2004). In legumes, seedling and developmental stages are more sensitive to salinity stress than the germination stage (Al-Mutawa et al. 2003).

Salinity also has an adverse effect on shoot biomass, pod set and pod filling in chickpea (*Cicer arietinum*), causing reduced yields (Flowers et al. 2010; Atieno et al. 2017). High salt concentration reduces the NO_3 supply from the soil which leads to lower protein content in chickpea, faba bean and mung bean (Cordovilla et al. 1995; Ghassemi-Golezani et al. 2010; Qados et al. 2011).

Conventional breeding approach has been widely used to develop stress tolerant and high yielding crop plants but this procedure is lengthy, labour intensive and costly and dependent on access to germplasm with enough genetic variability (Ashraf, 2010; Yu et al. 2016., Wani et al. 2016). To resolve these barriers associated with conventional breeding, biotechnological approaches such as genetic engineering provide a viable alternative and are now becoming more widely used throughout the world to obtain better results in shorter time. Transgenic approach is effectively used by plant scientists to impart salinity tolerance in various crops which mainly includes integration of genes that encode compatible organic solutes, ion transport proteins and transcriptional factors for gene regulation (Ashraf et al. 2010). These genetic processes demand the arbitration of several types of crucial enzymes including helicases. Helicases are the proteins which play a role in unwinding of nucleic acids and can be categorized into three groups- RNA helicases, DNA helicases, and Chromatin Remodelers. Other group of signal perception and signaling related genes (Passricha et al. 2019a). The constitutive expression of such genes can be used to construct stress tolerant plants. Studies reported that these genes provide stress tolerance when overexpressed are PDH45 (Shivakumara et al. 2017), p68 (Tuteja et al. 2014) and more (Passricha et al. 2019b). LecRLK homolog from *Pisum sativum* has been reported to provide salinity stress tolerance in overexpressed tobacco and rice plants (Passricha et al. 2019b; Vaid et al. 2013). In this review, we have summarized the functional validation of signal perception gene *Os*LecRLK, helicase gene and p68 gene in providing salinity stress tolerance in legume crops through transgenic approach.

Role of helicases in salt stress tolerance

DNA helicases are involved in replication, transcription, recombination, and repair so can also be called as ‘genome caretakers’ (Chu and Hickson, 2009; Brosh et al. 2013). On the other hand, RNA helicases play diverse roles in almost all processes of RNA metabolism like transcription, translation, pre-RNA splicing and export, removal of secondary structure of RNA ribosome biogenesis, miRNA processing and RNA metabolism which are crucial to cell survival (Putnam and Jankowsky, 2013; Jarmoskaite and Russell, 2014; Bourgeois et al. 2016; Sloan and Bohnsack, 2018). Chromatin remodelers perform ATP hydrolysis alter the interaction between DNA and histone proteins in a non-covalent fashion (Clapier and cairns, 2009). Out of the six helicase SFs, the monomeric SF1 and SF2 contain DNA helicases which are involved in the transcription, repair and recombination. Whereas SF6 contains replicative eukaryotic DNA helicases. Different abiotic stresses such as heavy metals, drought, salt, temperature, UV, etc. increase the amount of endogenous ROS in the cell which cause oxidative damage to the plant (Manova and Gruszka, 2015). These damages may end up in double-stranded breaks (DSBs), base modifications by insertion or deletions, inter - or - intra strand cross-linking or and formation of pyrimidine dimers. As plant cannot readily escape from the harsh climatic changes, they rely heavily on DNA damage detection and repair pathways for the timely and accurate removal of DNA lesions and preservation of genomic stability (Manova and Gruszka, 2015).

RuvB is a DNA helicase, which belongs to the AAA+ family of proteins and is very well characterized in bacteria. Almost all the members of this family are ATPases, but some members of this family contain helicase activity also. In prokaryotes, it plays a role in DNA damage repair mechanism by the formation of Holliday junction with RuvA and RuvC (Donaldson et al. 2004), branch migration and resolution of Holliday junction. A mutation study in *Saccharomyces cerevisiae* has revealed that RuvB is essential for growth of cells (Ahmad et al. 2012). It is an important component of various multiprotein complexes and is involved in multicellular pathways such as cell cycle progression, replication fork reversal, nonsense-mediated mRNA decay, apoptosis, mitosis, and development (Ahmad et al. 2012). *RuvB* stands as a potential candidate gene which can be involved in abiotic stress tolerance. Till date there are only two reports on characterization of RuvB in plants (Wang et al. 2011; Saifi et al. 2018). However, there are reports on other helicases like pea DNA helicase 45 (PDH45) which was found to be induced in pea seedlings in response to high salt (NaCl), dehydration, wounding, and low temperature. Transfer of its gene to tobacco provided a high salinity tolerance without affecting yield (Sanan-Mishra et al. 2005). Another helicase from pea (PDH47, pea DNA helicase 47) was reported to be induced in response to cold and salinity stress in shoots and roots, and heat and abscisic acid (ABA) treatment in roots (Vashisht et al. 2005). These reports suggest that helicases play an important role in stress tolerance. Though the exact mechanism of helicase-mediated tolerance of stress has not yet been understood. Saifi et al. (2018) highlights the role of rice homologue of RuvB gene (*OsRuv*BL1a) under various abiotic stresses. The *OsRuv*BL1a protein was characterized using *in silico* and biochemical approaches. The studies confirmed the presence of all the four characteristic motifs of AAA+ superfamily in this protein. It was also shown that *OsRuv*BL1a exhibits unique DNA-independent ATPase activity and unwinds the duplex DNA in the 3' to 5' direction. Moreover, the upregulation of its transcript under abiotic stress conditions suggested its involvement in multiple cellular pathways. Singh et al. (2020) developed transgenic pigeon pea lines having *Os*RuvB gene (Kharb et al. 2018; patent application number: 201811012099) and subjected six T_1 generation transgenic lines to 75mM salt stress. Observations were recorded for different physio-biochemical parameters viz. chlorophyll content, relative water content, MDA content, membrane injury index, total soluble sugar content, proline content, peroxidase activity, and catalase activity at 4 and 8 DAT with 3 replications for each treatment. The results showed that in addition to more chlorophyll and relative water content under salinity, the transgenic plants also showed higher activity of peroxidase and catalase. Level of proline and total soluble sugar was increased in T_1 transgenic plants, but the increase was lower than in wild type plants under salt stress. The transgenic lines didn't have significant increase in osmolytes proline and total soluble sugar, which indicates that the tolerance is being imparted either by some other osmolytes or some entirely different mechanism yet to be uncovered might be working in these plants.

*OsRuv*B gene was integrated in chickpea (cv. HC-1) plants using tissue culture independent patented protocol (Kharb et al. 2012) by Preeti and Kharb (2020) and obtained transformation efficiency of 17% when screening was done using gene specific primers. Transgene copy number in each event was detected by Southern hybridization which was later confirmed by real time PCR. After 20 days of germination plants were subjected to 100mM salt stress and it was observed that all the transgenic chickpea plants performed far better in comparison to wild type chickpea plants in terms of having high chlorophyll content, relative water content, proline content, total soluble sugar content, peroxidase and catalase activity but reduced MDA content and membrane injury index.

Role of p68 gene (DEAD-box family protein) in salt stress tolerance

DEAD-box helicases are required mostly in all aspects of RNA and DNA metabolism and play a significant role in various abiotic stresses, including salinity. The p68 is member of DEAD-box family and plays a very important role in cell/organ development (Stevenson et al. 1998). It also participates in various biological processes including pre-rRNA processing (Liu, 2002; Bates et al. 2005; Fuller-Pace, 2006), RNA-induced gene silencing (Ishizuka et al. 2002), transcription initiation (Fuller-Pace 2006) and alternative splicing processes (Kar et al. 2011). It was also reported that ATPase activity of recombinant p68 in yeast was stimulated by double-stranded RNA and it unwinds RNA in both 3' to 5' and 5’ to 3’ directions (Huang and Liu 2002). It has been reported that p68 RNA helicase activities are stimulated after phosphorylation with protein kinase C (Pradhan et al. 2005) which is a general cascade to cope with stresses in plants. Wang et al. (2013) reported that p68 also interacts with Ca^{2+}-CaM which regulates various signaling pathways leading to tolerance in plants under stress.

Psp68 DEAD-box protein exhibits ATPase activity in the presence of both DNA and RNA, binds to DNA as well as RNA and shows unique bipolar DNA helicase activity which suggest that it could be a multifunctional protein (Tuteja et al. 2014). *Psp68* provided salinity stress tolerance in transgenic tobacco and transgenic rice by reducing oxidative stress and improving photosynthesis machinery (Banu et al. 2015). Karthik et al. (2019) evidenced the role of the p68 gene against salinity, by enhancing the tolerance towards salinity stress in soybean plants. The transgenic soybean (T$_1$) plants showed a higher accumulation of chlorophyll, proline, CAT, APX, SOD, RWC, DHAR and MDHAR than the NT plants under salinity stress conditions. The transformed (T$_1$) soybean plants also retained a higher net photosynthetic rate, stomatal conductance and CO$_2$ assimilation as compared to NT plants. Further analysis revealed that (T$_1$) soybean plants accumulated higher K$^+$ and lower Na$^+$ than NT plants. Yield performance of transformed soybean plants was estimated in the transgenic greenhouse under salinity stress conditions. The transformed (T$_1$) soybean plants expressing the p68 gene were morphologically similar to non-transformed plants and produced 22–24 soybean pods/plant containing 8–9 g (dry weight) of seeds at 200 mM NaCl concentration.

Moreover, Banu et al. (2015) suggested that *Psp68* interacts with pea argonaute (AGO1), a catalytic component of the RNA-induced silencing complex (RISC) responsible for the gene silencing. The microarray analysis showed that *Psp68* regulates many transcripts involved in the abiotic and oxidative stress responses as well as gene-silencing mechanisms in rice. Thus, the *Psp68* functions as a molecular switch in different signaling path-ways leading to stress tolerance. Overall, *P*sp68 may serve as a useful biotechnological tool for the improvement of stress tolerance crop.

Neha and Kharb (2019) obtained 16% transformation efficiency when transformed pigeon pea (cv. Manak) with *Psp68* gene (Fig.1). Selected PCR positive transgenic plants were subjected to 75mM NaCl salt stress 15 days after germination and observance were recorded 4 days and 8 days after treatment. Analysis of various physio-biochemical parameters showed that transgenic plants performed well with respect to all the parameters with higher chlorophyll content, relative water content, total soluble sugar content, proline content, catalase and peroxidase activity and reduced lipid peroxidation, electrolyte leakage.

Role of *OsLecRLK* in salt stress tolerance

Lectin receptor-like kinase (LecRLK) is an important family that plays a major role in stress sensing through lectin receptor and further activates downstream signaling by kinase domain.

Plant perceives stress by various sensors (Wall-associated kinase, G-protein couple receptors-like protein(s) or receptor like kinases [RLKs]) present on the cell membrane, which leads to activation of downstream signaling (Tuteja and Sopory, 2008). Plant lectin receptor-like kinases (LecRLKs) are membrane-embedded RLK proteins. Extracellular lectin domain has a role in stress perception through recognition of different ligands (such as hormones and complex saccharides) generated in response to environmental challenges (Barre et al. 2002; Passricha et al. 2019b). RLKs participate in various processes, including regulation of development, disease resistance, and hormone perception. RLK is a vast family of proteins that have 610 genes in *Arabidopsis* and 1100 genes in rice (Morillo and Tax 2006). Members of this gene family are not well characterized but some reports provide their role in stress such as *Arabidopsis*, L-VI.2 (At5g01540) provide resistance against *Pseudomonas syringae* and *Pectobacterium carotovorum* (Singh et al. 2012). LecRLK-1 in *Nicotiana tabacum* which is responsive to herbivorous signaling mediated through elicitors released by larvae of *Medunca sexta* (Bonaventure, 2011). NbLRK1 (LecRLK in *Nicotiana benthamiana*) directly associate with elicitor protein IFN1 released *by Phytophthora infestans* through its kinase domain (Kanzaki et al. 2008). Similar studies on LecRLK in different plant systems such as *Arabidopsis* (Deng et al. 2009*), Pisum sativu*m (Vaid et al. 2012), Glycine soja (Sun et al. 2013) and rice (Saifi et al. 2017) showed the importance of LecRLKs in alleviating stress condition. Among the 610 RLKs in *Arabidopsis thaliana* and 1100 RLKs in rice (Shiu et al. 2004), some have been characterized as receptors for polypeptides, phytohormones and pathogens. Each of these RLKs can rapidly initiate signalling through the formation of oligomers and cross-phosphorylation of the intracellular serine/threonine kinase domain upon binding to ligand (Dievart and Clark 2004) and together they play diverse roles in plant development and resistance (Antolín-Llovera et al. 2012; Osakabe et al. 2013). Some RLKs are also reported to play role in drought and salt responses (Ouyang et al. 2010; Marshall et al. 2012; Vaid et al. 2013). Rice SIK1 (Os06g03970), that is expressed most strongly in stem and panicle but which is not expressed in root, was found to be salt-inducible and a positive regulator of salt tolerance (Ouyang et al. 2010).

The *LecRLKs* acting as membrane receptors is well-known, however related evidences of downstream and upstream components and how they interact with various signalling components is still not known.

Vaid et al. (2015) reported that *PsLecRLK* transcripts are upregulated in salinity stress response and overexpression of the gene showed enhanced water uptake in plants through the activation of water channel. In 2014, *SIT1* a salt tolerance gene reported mainly expressing in root epidermal cells in rice. The gene was found rapidly activated by NaCl and phosphorylated MPK3/6 then further facilitates ethylene accumulation & ROS production and accumulation in plants ultimately leads to inhibition of plant death under stress. Zhang et al. (2019) reported *PnRLK-1* (a type of cytoplasmic RLK) in an Antarctic moss (*Pohlia nutans*) upturn ABA sensitivity and also upregulates ROS scavenger machinery that suppress ROS accumulation that ultimately results in reduction of salt stress. Table 1 depicts impact of various transgenes inserted in different plants.

Pratibha (2019) transformed pigeon pea (cv. Manak) plants with *OsLecRLK* gene and obtained 16 plants out of 86 showing amplification for the gene of interest representing a transformation efficiency of 18.6%. Transgenic copy no. and integration was confirmed through Southern hybridization and Real-time PCR analysis in T_0 generation and found that 5 Transgenic lines (L-9, L-17, L-18, L-19 and L-48) carried single copy insertion of gene whereas, one transgenic line (L-89) with two copies of the transgene. Physio-biochemical analysis was done to assess the efficacy of transgene via subjecting wild type and selected T_1 transgenic plants to 75 mM salt stress. The results showed that transgenic line performed better in terms of maintaining higher relative water content, chlorophyll content, total soluble sugar content, proline content, peroxidase and catalase activity in comparison to the wild type plants. Moreover, membrane injury index and MDA content were significantly reduced in transgenic lines then wild type plants indicating that the transgenic lines were less affected by salt stress (Figure 1)

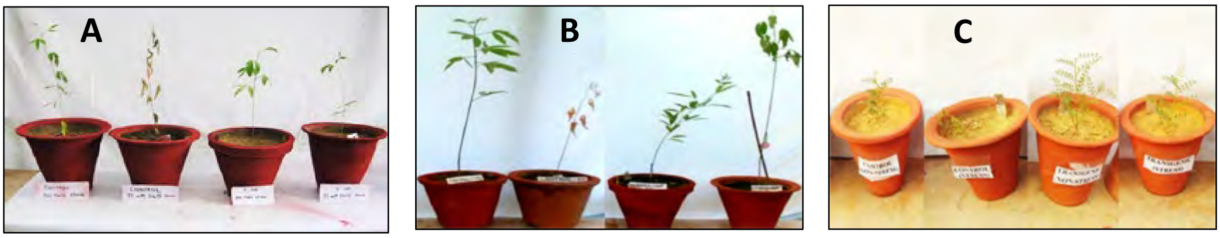
Chickpea (cv. HC-1) plants were transformed by Singh (2018) with *OsLecRLK* gene and obtained 17.82% transformation efficiency. Transgene copy number was confirmed using Real time PCR & Southern hybridization. Transgenic chickpea plants were subjected to 100 mM salinity stress 15 days after germination. The transgenic chickpea plants showed & better growth than non-transformed chickpea plants and synthesized more compatible solute such as proline, high sugar level, increased MDA content and decreased membrane injury and significant maintenance of chlorophyll content under salt stress conditions. (Figure 1). Table 2 shows comparative performance of various physio-biochemical parameters in different transgenic lines.

Conclusions

The present review highlights the helicases (*OsRuv*B and p68) and kinases (*OsLecRLK*) mediated salt stress tolerance in two legume crops i.e. chickpea and pigeon pea. It was also interesting to observe that genes isolated from rice, a monocot, induced salt tolerance in chickpea and pigeon pea, both being dicot plants. Although more research is required to identify the exact molecular mechanism and the underlying signalling pathway of all these above mentioned genes. Till date, no information on ligands, downstream targets or factors governing activation or inactivation of *OsRuv*B and *OsLecRLK* is available. Therefore, further research is being undertaken in our laboratory to understand the role of these genes in providing salinity tolerance in plants.

Acknowledgements

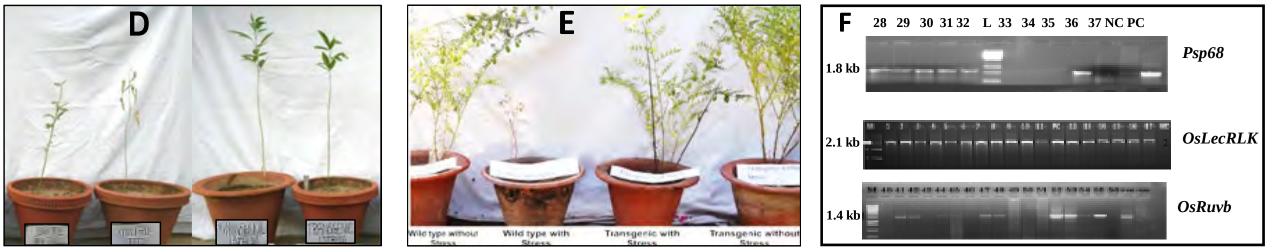
The authors are thankful to Prof N. Tuteja, ICGEB, New Delhi for providing the transgenes and CCSHAU Hisar for lab support during the project work.



Effect of 75 mM NaCl salt stress on wild type (control) and *OsRuv*B carrying transgenic pigeon pea plants. (Original)

Effect of 75 mM NaCl salt stress on wild-type and *p68* gene containing transgenic pigeon pea plants. (Original)

Effect of 100 mM NaCl salt stress on wild-type *OsRuv*B containing transgenic chickpea plants. (Original)



Effect of 75 mM NaCl salt stress on wild type (control) and *OsLec-RLK* carrying transgenic pigeon pea plants. (Original)

Effect of 100 mM NaCl salt stress on wild-type and transgenic carrying *OsLec-RLK* gene chickpea plants. (Original)

PCR amplification of *OsRuv*B, *p68* and *LecRLK* gene using gene specific primers.

Figure 1. Effect of salt stress on transgenic chickpea and pigeon pea carrying *Os*RuvB, *Psp68* and *Os*LecRLK gene (A-E) and PCR amplification of *Os*RuvB, *Psp68* and *Os*LecRLK genes in transgenic pigeon pea and chickpea plants.

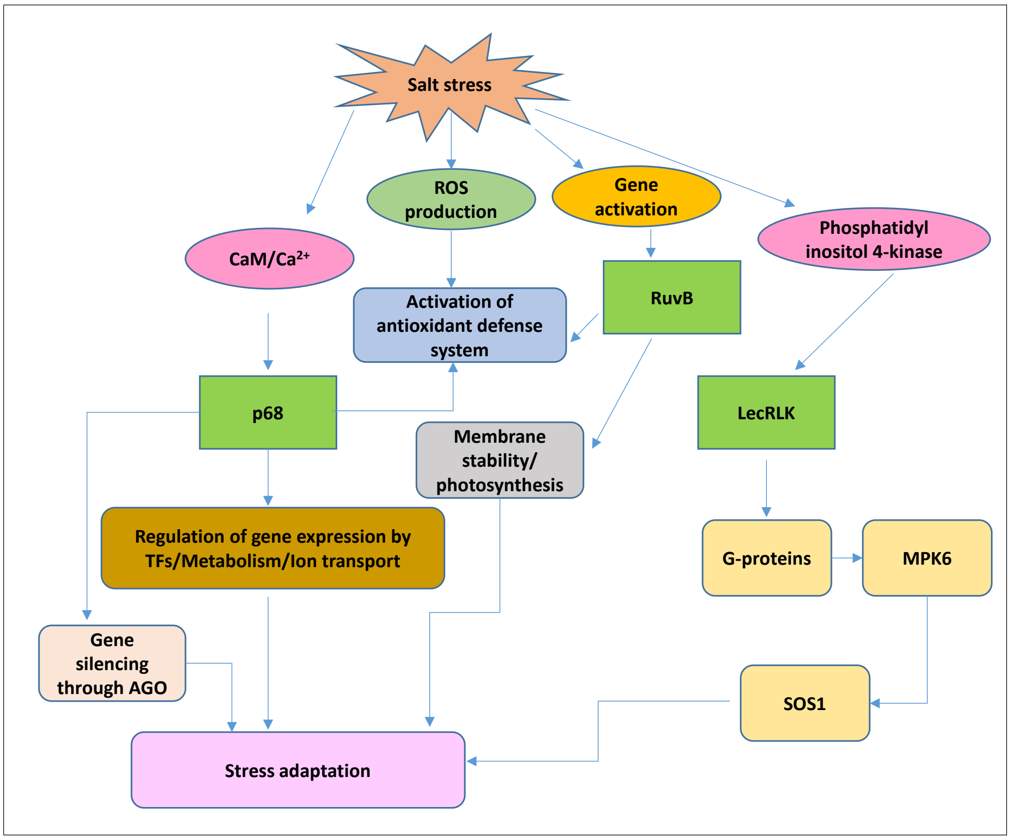


Figure 2. Proposed action of helicases (*RuvB* and *p68*) and lectin receptor kinases (*Lec-RLK*) in legume for providing salinity tolerance.

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