Linking Duplication of a Calcium Sensor to Salt Tolerance in *Eutrema salsugineum*1[OPEN]

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The SALT-OVERLY-SENSITIVE (SOS) pathway in Arabidopsis (*Arabidopsis thaliana*) functions to prevent the toxic accumulation of sodium in the cytosol when plants are grown in salt-affected soils. In this pathway, the CALCIUNEURIN B-LIKE10 (AtCBL10) calcium sensor interacts with the AtSOS2 kinase to activate the AtSOS1 plasma membrane sodium/proton exchanger. CBL10 has been duplicated in Eutrema (*Eutrema salsugineum*), a salt-tolerant relative of Arabidopsis. Because Eutrema maintains growth in salt-affected soils that kill most crop plants, the duplication of CBL10 provides a unique opportunity to functionally test the outcome of gene duplication and its link to plant salt tolerance. In Eutrema, individual down-regulation of the duplicated CBL10 genes (EsCBL10a and EsCBL10b) decreased growth in the presence of salt and, in combination, led to an even greater decrease, suggesting that both genes function in response to salt and have distinct functions. Cross-species complementation assays demonstrated that EsCBL10b has an enhanced ability to activate the SOS pathway while EsCBL10a has a function not performed by AtCBL10 or EsCBL10b. Chimeric EsCBL10a/EsCBL10b proteins revealed that the specific functions of the EsCBL10 proteins resulted from changes in the amino terminus. The duplication of CBL10 increased calcium-mediated signaling capacity in Eutrema and conferred increased salt tolerance to salt-sensitive Arabidopsis.

A growing challenge to food security is the loss of arable land due to the accumulation of salt in the soil. The estimated annual global cost of crop loss due to soil salinity is $27.3 billion (Qadir et al., 2014). The Food and Agricultural Organization estimates that 20% of irrigated land, which produces over 40% of the world’s food, is affected by salt and 1% to 2% of irrigated land is lost each year due to the accumulation of salt (FAO, 2002). Solutions for sustainable agriculture will require identification of genes and mechanisms that contribute to salt tolerance to enable exploitation of natural trait variation and the use of transgenic technologies.

Salt, particularly sodium chloride (NaCl), negatively affects plant growth in several ways. The accumulation of salt in the soil restricts water movement into the plant (osmotic stress) and the accumulation of sodium ions in a plant cell interferes with metabolic processes (ionic stress; Munns and Tester, 2008). Molecular studies in *Arabidopsis* (*Arabidopsis thaliana*) have identified genes important for alleviating ionic stress and provided insight into the pathways in which these genes function. For example, the Arabidopsis SALT-OVERLY-SENSITIVE (SOS) pathway removes sodium from the cell, preventing its toxic accumulation in the cytosol. In this pathway, accumulation of sodium triggers an influx of cytosolic calcium. Increased calcium is perceived by two calcium sensors, SOS3 (roots) and CALCIUNEURIN B-LIKE10 (CBL10, also known as SOS3-LIKE CALCIUM-BINDING PROTEIN8; leaves), which bind to and activate the SOS2 Ser/Thr protein kinase (also known as CBL-INTERACTING PROTEIN KINASE24 [CIPK24]; Liu and Zhu, 1998; Halfter et al., 2000; Liu et al., 2000; Kim et al., 2007; Quan et al., 2007). SOS2 phosphorylates SOS1, a plasma membrane sodium/proton exchanger, initiating transport of sodium out of the cell (Shi et al., 2000; Qiu et al., 2002, 2003; Quintero et al., 2002).

While advances have been made identifying genes important for the growth of Arabidopsis in the presence of salt, Arabidopsis, like many crop plants, is sensitive to soil salinity (glycophytes; Munns and Tester, 2008). Genetic variability for plant salt tolerance exists, with some plants maintaining growth longer in high concentrations of salt (halophytes). Presently, little is known about the mechanisms underlying halophyte adaptation to salinity (Munns and Tester, 2008). *Eutrema* (*Eutrema salsugineum*; formerly *Thellungiella halophila*), a small crucifer native to the seashore saline soils of Eastern China, is a halophytic relative of Arabidopsis (Bressan et al., 2001; Inan et al., 2004; Amtmann, 2009). *Eutrema* is emerging as a model for...
understanding plant adaptation to soil salinity (Inan et al., 2004; Yang et al., 2013). Eutrema does not require salt for optimal growth but is able to survive in conditions that kill most, if not all, crop plants and its tolerance does not appear to rely on specialized morphological structures (Inan et al., 2004; Amtmann, 2009).

Comparative genomic studies have revealed that plant gene families are largely conserved across species, indicating that plants amplify and modify a basic set of genes rather than developing new species-specific gene families (Flagel and Wendel, 2009). This tendency to use a basic suite of genes suggested that the SOS pathway, identified in Arabidopsis, likely functions in Eutrema with modifications that contribute to its increased salt tolerance. Several studies have compared the activity of the SOS1 sodium/proton exchanger in Arabidopsis (Saccharomyces cerevisiae) and Eutrema (EsSOS1). When each protein was expressed in a salt-sensitive strain of yeast (Saccharomyces cerevisiae), yeast expressing EsSOS1 grew better in the presence of salt than yeast expressing AtSOS1, indicating that EsSOS1 has a greater level of self-activation than AtSOS1 (Oh et al., 2009; Jarvis et al., 2014). When the entire SOS pathways were reconstructed in this salt-sensitive yeast strain, yeast expressing the Eutrema SOS pathway (EsSOS3, EsSOS2, and EsSOS1) were more salt tolerant than yeast expressing the Arabidopsis SOS pathway (AtSOS3, AtSOS2, and AtSOS1; Jarvis et al., 2014). While it was not possible to determine whether the greater growth of yeast expressing the Eutrema SOS pathway was due to EsSOS1 alone or enhanced activation of EsSOS1 by EsSOS2 and EsSOS3, this study links the Eutrema SOS pathway to salt tolerance. Based on the importance of the SOS pathway in salt tolerance and its well-established link to calcium signaling, additional putative Eutrema SOS pathway genes were identified and characterized. One, CBL10 (EsCBL10), was found to be duplicated (EsCBL10a and EsCBL10b) and is the focus of this study.

Gene duplication is a major source of genetic diversity and, consequently, adaptive evolution (Ohno, 1970; Kondrashov, 2012). The maintenance of duplicated gene pairs in a genome can be indicative of an adaptive benefit conferred by the paralogous genes (Hahn, 2009; Kondrashov, 2012). Because Eutrema maintains growth in salt-affected soils that kill most crop plants, the duplication of CBL10 provides a unique opportunity to functionally test the outcome of gene duplication and its link to plant salt tolerance. To understand the roles the duplicated Eutrema CBL10 genes play in response to salinity, we monitored their expression patterns and reduced their expression individually and in combination. To determine the extent of divergence in CBL10 function after duplication and to uncover elements of the signaling pathways in which the proteins function, protein activities were examined using cross-species complementation and yeast two-hybrid assays. To identify domains and amino acids responsible for the divergence in function, chimeric EsCBL10 proteins were generated. We found that, in Eutrema, down-regulation of either of the duplicated EsCBL10 genes decreased growth in the presence of salt, suggesting that both genes function in response to salinity. Down-regulation of both EsCBL10 genes in combination led to an even greater decrease in growth, suggesting the genes have additive effects or different functions. Based on cross-species complementation assays, we found that EsCBL10b has an enhanced ability to activate the SOS pathway while EsCBL10a has a function not performed by Arabidopsis CBL10 (AtCBL10) or EsCBL10b. Four kinases that interact with EsCBL10a were identified, and chimeric proteins revealed that the role of EsCBL10b in the SOS pathway and the role of EsCBL10a in an alternative pathway were the result of changes in the N termini of the proteins. Analysis of the EsSOS3 calcium sensor revealed that EsCBL10a and EsSOS3 have partially overlapping functions but also distinct roles in salt tolerance. The duplication of EsCBL10 appears to have increased the calcium-mediated signaling capacity in Eutrema and the duplicated genes conferred increased salt tolerance when expressed in salt-sensitive Arabidopsis.

RESULTS

Eutrema Is a Salt-Tolerant Relative of Arabidopsis

To compare salt tolerance in Arabidopsis and Eutrema, growth of the two species was monitored in the absence and presence of salt during vegetative and reproductive development. Vegetative (rosette) growth in Arabidopsis was reduced at all salt concentrations while Eutrema maintained rosette growth even in the presence of 300 mM NaCl (Fig. 1A). During reproductive development, Arabidopsis inflorescence number, height, and seed production were reduced at all salt concentrations, while only small reductions in these traits were found in Eutrema (Fig. 1B). These results, in combination with data from previous studies (Bressan et al., 2001; Inan et al., 2004), demonstrate that Eutrema is more salt-tolerant than Arabidopsis during both vegetative and reproductive development. Subsequent studies focused on understanding if the duplicated EsCBL10 genes play a role in this salt tolerance.

The Duplicated EsCBL10 Genes Play a Role in Eutrema’s Response to Salt

Models describing the retention of duplicated genes predict changes in expression and/or gene function (Flagel and Wendel, 2009; Hahn, 2009; Inan and Kondrashov, 2010). To uncover differences between EsCBL10a and EsCBL10b, transcript accumulation was examined and expression of the genes was reduced individually and in combination. In Arabidopsis, CBL10 is alternatively spliced into two primary transcripts: one encoding a functional calcium-binding protein and
one encoding a nonfunctional protein (cannot complement the Atcbl10 mutant) likely due to a premature stop codon in a retained intron (Supplemental Fig. S1; Quan et al., 2007). Reverse transcription-PCR was used to determine the expression patterns of the Eutrema CBL10 genes and to identify alternatively spliced transcripts. Expression of EsCBL10b in Eutrema was most similar to the expression of AtCBL10 in Arabidopsis; both transcripts were found in aerial tissues (Fig. 2). When comparing transcript accumulation in leaves and flowers, the EsCBL10b transcript was present at similar levels in both organs whereas the AtCBL10 transcript was found predominantly in leaves and only weakly in flowers (Fig. 2). EsCBL10a was expressed throughout Eutrema, with similar transcript levels in roots, leaves, and flowers (Fig. 2). Structures of the EsCBL10 splice variants were determined by cloning and sequencing 10 transcripts from each band; multiple variants with different splicing patterns were identified (Supplemental Fig. S1). If translated, the majority of these variants would likely encode nonfunctional proteins due to premature stop codons or removal of domains required for binding calcium (Supplemental Fig. S1). EsCBL10a and EsCBL10b transcripts whose exon/intron structures were most similar to AtCBL10 and likely encode functional proteins (listed first in Supplemental Fig. S1) were used in experiments described below.

To determine if the EsCBL10 duplication contributes to salt tolerance, six artificial microRNAs (amiRNAs) were designed to reduce expression of EsCBL10a and EsCBL10b individually and in combination (Supplemental Fig. S2). Only one of the two EsCBL10a amiRNAs was specific, the other reduced transcript levels of both genes. The salt tolerance of nine independently transformed, single insertion, homozygous lines for each target gene was monitored. Two representative lines were chosen: one line per amiRNA
construct except for \( \text{EsCBL10a} \), for which only one amiRNA construct was used. Seedling weight and root length of seedlings grown on solid medium were measured as likely components of salt tolerance (shoot biomass or yield) to allow small growth differences associated with down-regulation of one or both genes to be measured. Reduced expression of \( \text{EsCBL10a} \) and \( \text{EsCBL10b} \) individually and in combination did not affect growth in control conditions but decreased growth in the presence of salt (Fig. 3, A and B). Compared with the wild type, in amiRNA lines targeting \( \text{EsCBL10a} \), transcript accumulation was reduced (56% and 32%), resulting in seedling hypersensitivity to salt (23% and 21% decrease in fresh weight; Fig. 3, B and C). In amiRNA lines targeting \( \text{EsCBL10b} \), transcript accumulation was reduced (45% and 61%), resulting in seedling hypersensitivity to salt (23% and 22% decrease in fresh weight; Fig. 3, B and C). In amiRNA lines targeting both genes, transcript accumulation of \( \text{EsCBL10a} \) and \( \text{EsCBL10b} \) was reduced (64% and 36% and 61% and 42%, respectively), resulting in even greater hypersensitivity to salt (63% and 45% decrease in fresh weight; Fig. 3, B and C).

When grown in the presence of salt, lines with reduced expression of both \( \text{EsCBL10a} \) and \( \text{EsCBL10b} \) had fewer branch roots (77% and 67% decrease) and shorter primary roots (43% and 26% decrease) compared with the wild type (Supplemental Fig. S3, A and B). The effect of salt on root development in lines targeting \( \text{EsCBL10a} \) or \( \text{EsCBL10b} \) individually was variable (Supplemental Fig. S3, A and B).

These results suggest that \( \text{EsCBL10a} \) and \( \text{EsCBL10b} \) function in Eutrema’s response to salt and may have both overlapping and distinct functions. To provide additional insight into the inherent biochemical activities of the duplicated proteins and to determine whether differences in function have arisen through alterations in the amino acid sequence, in vivo, cross-species complementation assays in single mutant genetic backgrounds were performed.

\( \text{EsCBL10a} \) and \( \text{EsCBL10b} \) Share \( \text{AtCBL10} \) Function

To determine if the Eutrema \( \text{AtCBL10} \) genes are functional orthologs of \( \text{AtCBL10} \), the coding sequence of each gene was expressed in the Arabidopsis \( \text{cbl10} \) mutant \( (\text{Atcbl10}) \) downstream of a constitutive promoter. The \text{Cauliflower mosaic virus 35S (CaMV 35S)} promoter was used to ensure that the coding sequence was strongly expressed without the presence of introns, which are necessary for full expression when using the native promoter and which might lead to alternative splicing. The salt tolerance of five independently transformed, single insertion, homozygous lines was assessed for each gene. Both \( \text{EsCBL10a} \) and \( \text{EsCBL10b} \) complemented the \( \text{Atcbl10} \) salt-sensitive phenotype in all lines examined; however, the degree of complementation varied relative to the growth of wild-type Arabidopsis. Strongly complementing lines had higher transcript accumulation and fully restored growth to wild-type levels (Fig. 4). Weakly complementing lines had lower transcript accumulation and restored growth to 45% of that in the wild type (Fig. 4). Representative strong and weak lines for each are shown (Fig. 4). In Arabidopsis, loss of \( \text{CBL10} \) also results in a fertilization defect with unfertilized ovules (Monihan et al., 2016). Both \( \text{EsCBL10a} \) and \( \text{EsCBL10b} \) restored fertility in the \( \text{Atcbl10} \) mutant grown in the presence of salt (Supplemental Fig. S4). These results demonstrate that there is conservation of \( \text{CBL10} \) function in Arabidopsis and Eutrema.

\( \text{EsCBL10a} \) and \( \text{EsCBL10b} \) Function in Distinct Pathways in Response to Salt

In Arabidopsis, \( \text{CBL10} \) functions in the SOS pathway to prevent the toxic build-up of sodium in the cytosol (Quan et al., 2007; Lin et al., 2009). Potential roles for \( \text{EsCBL10a} \) and \( \text{EsCBL10b} \) in this pathway were investigated using a salt-sensitive strain of yeast (\text{AXT3K}) expressing \( \text{SOS2} \) and \( \text{SOS1} \) from Arabidopsis or Eutrema. In AXT3K cells expressing either pathway, salt tolerance was greatest in those cells expressing \( \text{EsCBL10b} \) and weakest in those expressing \( \text{EsCBL10a} \) (Fig. 5, A and B); differences in activation were not due to differences in gene expression (Fig. 5C). Enhanced activation of the SOS pathway by \( \text{EsCBL10b} \) could be due to stronger interaction with \( \text{AtSOS2} \); yeast two-hybrid assays were performed to determine the strength of this interaction. \( \text{EsCBL10b} \) and \( \text{AtCBL10} \) interacted more strongly with \( \text{AtSOS2} \) than \( \text{EsCBL10a} \) (Fig. 6) and interaction was orientation-specific (Supplemental Fig. S5).
Because EsCBL10a functions in plant responses to salt (down-regulation in Eutrema results in increased salt sensitivity and expression complements the \textit{Atcbl10} salt-sensitive phenotype) but only weakly activates the SOS pathway, we determined if EsCBL10a can function in alternative pathways. Even though \textit{AtCBL10} and \textit{AtSOS3} both activate the SOS pathway, they do not reciprocally complement the \textit{Atcbl10} or \textit{Atsos3} salt-sensitive phenotypes, indicating that the two proteins also function in distinct pathways (Quan et al., 2007). To determine if \textit{EsCBL10a} and \textit{EsCBL10b} can function in an Arabidopsis SOS3 pathway, we determined if \textit{EsCBL10a} can function in alternative pathways. Even though \textit{AtCBL10} and \textit{AtSOS3} both activate the SOS pathway, they do not reciprocally complement the \textit{Atcbl10} or \textit{Atsos3} salt-sensitive phenotypes, indicating that the two proteins also function in distinct pathways (Quan et al., 2007). To determine if \textit{EsCBL10a} and \textit{EsCBL10b} can function in an Arabidopsis SOS3 pathway, the genes were expressed in \textit{Atsos3} downstream of the CaMV 35S promoter and the salt tolerance of five independently transformed, single insertion, homozygous lines was determined for each gene. \textit{EsCBL10a} but not \textit{EsCBL10b} complemented the \textit{Atsos3} salt-sensitive phenotype in all lines examined; two representative lines of each are shown (Fig. 7, A and B). Expression of \textit{EsCBL10a} in \textit{Atsos3} restored rosette growth (fresh weight) to wild-type levels and partially restored root growth (Fig. 7B). Transcript accumulation of \textit{EsCBL10a} and \textit{EsCBL10b} was similar, suggesting that the inability of \textit{EsCBL10b} to complement the mutant was not due to lack of gene expression (Fig. 7C).

The ability of \textit{EsCBL10a} to complement \textit{Atsos3} in combination with its weaker interaction with \textit{AtSOS2} suggested that \textit{EsCBL10a} may function with a different kinase. In Arabidopsis, SOS2 belongs to the 26-member CIPK family, raising the possibility that \textit{EsCBL10a} might interact with another CIPK to complement the \textit{Atsos3} salt-sensitive phenotype. Yeast two-hybrid assays were performed with all 26 CIPK proteins to identify a kinase that interacts specifically with \textit{EsCBL10a} but not with \textit{EsCBL10b} or \textit{AtCBL10}. Four CIPK kinases (AtCIPK13, AtCIPK16, AtCIPK6, and

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure3.png}
\caption{Reduced expression of \textit{EsCBL10a} and \textit{EsCBL10b} results in seedling hypersensitivity to salt. AmiRNAs were designed to reduce expression of \textit{EsCBL10a} (\textit{E10a}) and \textit{EsCBL10b} (\textit{E10b}) individually (a1 and b1 and b2, respectively) and in combination (ab1 and ab2) and growth in the absence (Control) and presence of salt (200 mM NaCl) was monitored. WT, Wild type; -, no amiRNA construct. A, Photographs of wild type and amiRNA lines. Bar = 1 cm for all images. B, Fresh weight was measured to quantify growth. Data are means ± se of at least 30 seedlings per genotype grown in three independent experiments. C, Reverse transcription quantitative PCR. mRNA levels were normalized to \textit{EsACTIN2} (\textit{DCT}) and the fold change in expression relative to the wild type was calculated (2^{-\Delta\DeltaCT}). Data are means ± se of three biological replicates with two technical replicates each. For all graphs, different letters indicate significant differences between genotypes (two-way ANOVA, Tukey-Kramer honestly significant difference [HSD], \(P \leq 0.05\)).}
\end{figure}
AtCIPK18) specifically interacted with EsCBL10a (Fig. 8). In the opposite orientation, interaction was not specific (Supplemental Fig. S6). Taken together, these results suggest that interaction with different protein partners might underlie the differences in EsCBL10a and EsCBL10b function.

The EsCBL10a- and EsCBL10b-Specific Functions Reside in the Amino Terminus

Identification of amino acids or domains that underlie specific functions is critical for understanding how duplication of a gene may contribute to plant adaptation. Because the same promoter was used for all EsCBL10a and EsCBL10b constructs, their distinct functions likely arose through alterations in biochemical functions via changes in their amino acid sequences. In AtCBL10, the hydrophobic domain has been linked to membrane localization, the four EF-hand domains bind calcium, and a Ser in the carboxy terminus of the protein can be phosphorylated to strengthen interaction with AtSOS2 (Supplemental Fig. S7; Quan et al., 2007; Lin et al., 2009). Based on crystal structures, it has been suggested that CBL proteins interact with CIPK proteins through the three-dimensional arrangement of the EF-hand domains, which form a hydrophobic pocket to interact with the CIPK-FISL/NAF domain (a 24-amino acid CIPK-specific domain; Guo et al., 2001; Sánchez-Barrena et al., 2005; Akaboshi et al., 2008). Alignment of the EsCBL10 and AtCBL10 proteins identified two additional regions in the amino terminus that may be important for function: an insertion of seven amino acids in EsCBL10a and a variable region in which the amino acid sequence is distinct in all three CBL10 proteins (Supplemental Fig. S7). To identify amino acids or domains that underlie the distinct functions of the EsCBL10 proteins, portions of EsCBL10a and EsCBL10b were exchanged to generate three sets of chimeric proteins (Supplemental Fig. S7). The first chimeric proteins fused the amino-terminal half of one of the EsCBL10 proteins with the carboxy-terminal half of the other (Supplemental Fig. S7). The second and third sets of chimeric proteins progressively narrowed down the region underlying the specific functions (Supplemental Fig. S7). To verify that the chimeric proteins are functional, each protein was expressed in the Atcbl10 mutant downstream of the CaMV 35S promoter and the salt tolerance of five independently transformed, single insertion, homozygous lines was determined; all chimeric proteins complemented the Atcbl10 salt-sensitive phenotype (Supplemental Figs. S8 and S9). To identify regions important for activation of the SOS pathway, the chimeric proteins were expressed...
in AXT3K along with AtSOS2 and AtSOS1 and growth in the presence of salt was assessed as an indication of pathway activity. Based on these experiments, the ability of EsCBL10b to strongly activate the SOS pathway resides in the first eight amino acids of the protein (Fig. 9). To identify regions important for complementation of Atsos3, the chimeric proteins were expressed in the Atsos3 mutant downstream of the CaMV 35S promoter and the salt tolerance of five independently transformed, single insertion, homozygous lines was determined (Supplemental Fig. S8C). All chimeric proteins with the EsCBL10a hydrophobic domain (aN1, aN2, and bN3) complemented the Atsos3 salt-sensitive phenotype like the full-length protein (major element; Fig. 10). Like EsCBL10a, the chimeric protein bN2, which has the EsCBL10b hydrophobic domain and the EsCBL10a variable region, complemented Atsos3 rosette growth (fresh weight) but not root length (minor element; Fig. 10).

EsCBL10a and EsSOS3 Function in Distinct Pathways in Response to Salt

The ability of EsCBL10a to complement the Atsos3 salt-sensitive phenotype suggests that two genes in Eutrema (EsCBL10a and the AtSOS3 ortholog EsSOS3) may have similar functions. To compare the activities of the two proteins, the salt tolerance of Atsos3 seedlings expressing EsCBL10a or EsSOS3 was examined. EsCBL10a and EsSOS3 were found to complement Atsos3 in an organ-specific manner; EsCBL10a restored rosette growth (fresh weight) while EsSOS3 restored primary root growth to wild-type levels (Fig. 7, A and B). The ability of EsSOS3 and EsCBL10a to activate the SOS pathway was compared by expressing the genes in AXT3K with SOS2 and SOS1 from either Arabidopsis or Eutrema. EsSOS3 activated the Arabidopsis and Eutrema SOS pathways better than EsCBL10a but not as well as EsCBL10b (Supplemental Fig. S10). To determine the extent of EsSOS3’s role in salt tolerance, it was expressed in the Atcbl10 mutant and the salt tolerance of five independently transformed, single insertion, homozygous lines was determined. While EsSOS3 did not restore growth to wild-type or EsCBL10a levels, seedlings expressing EsSOS3 had longer roots and greater fresh weight than Atcbl10 seedlings (Fig. 4; Supplemental Fig. S11). The difference in the ability of EsCBL10a and EsSOS3 to complement Atsos3 and Atcbl10 and to activate the SOS pathway suggests that, while both play a role in response to salt, these calcium sensors have diverged in function.

The Eutrema Calcium Sensors Confer Salt Tolerance to Arabidopsis

Down-regulation of EsCBL10a and EsCBL10b, individually and in combination, decreased growth of Eutrema, indicating that both genes are necessary for salt tolerance. To determine if the duplicated genes are sufficient to confer salt tolerance, EsCBL10a and EsCBL10b were expressed in combination in Atcbl10, and EsCBL10a and EsSOS3 in combination in Atsos3. For these studies, the mutants were used instead of wild-type Arabidopsis to maintain gene composition consistent with what is found in Eutrema. Plants expressing two Eutrema calcium sensors in combination were generated by crossing homozygous plants expressing each gene individually and the salt tolerance of heterozygous seedlings was analyzed. For plants expressing EsCBL10a and EsCBL10b in combination, two crosses were generated using strongly complementing lines and two were generated...
using weakly complementing lines; a representative cross generated from strong lines is shown (Fig. 11). The majority of the crosses (three of four) were unable to confer salt tolerance above what was found in wild-type Arabidopsis or Atchbl10 expressing AtCBL10. In the fourth cross, expression of both genes improved growth relative to wild-type Arabidopsis in the presence of salt (growth increased by 10%; Fig. 11, A and B).

![Figure 6. EsCBL10b and AtCBL10 interact more strongly with AtSOS2 than EsCBL10a. AtCBL10 (A10), EsCBL10a (E10a), EsCBL10b (E10b), and AtSOS3 (A3) were fused to the GAL4 DNA-activation domain (AD) and interaction with AtSOS2 fused to the GAL4 DNA-binding domain (BD) was assessed using yeast two-hybrid assays. Serial decimal dilutions of diploid yeast harboring both constructs were spotted onto synthetic defined media (SD) minus Leu (L) and Trp (W), minus LW and His (H), minus LWH and adenine (A), or with the addition of 0.5 mM 3-amino-1,2,4-triazole (3AT). Two independently mated colonies were assayed in two biological replicates; one representative image is shown.](image)

![Figure 7. EsCBL10a but not EsCBL10b complements the Atsos3 salt-sensitive phenotype. EsCBL10a, EsCBL10b, and EsSOS3 were expressed in Atsos3 and growth in the absence (Control) and presence of salt (75 mM NaCl) was monitored. A, Photographs of the wild type (WT), Atsos3, and Atsos3 expressing AtCBL10 (A10), EsCBL10a (E10a), EsCBL10b (E10b), AtSOS3 (A3), or EsSOS3 (E3). Bar = 1 cm for all images. B, Fresh weight and the length of the primary root were measured to quantify growth. Data are means ± se of at least 24 seedlings per genotype grown in three independent experiments. For all graphs, different letters indicate significant differences between genotypes (two-way ANOVA, Tukey-Kramer HSD, *P* ≤ 0.05). C, Transcript accumulation. ACTIN, Loading control. One representative image of three replicates is shown.](image)
These results suggest that the ability of \( EsCBL10a \) and \( EsCBL10b \) to confer salt tolerance is variable; however, coexpression can increase growth in the presence of salt above that of wild-type Arabidopsis.

In all four crosses expressing \( EsCBL10a \) and \( EsSOS3 \) in combination, seedlings had longer primary roots. A representative cross in which root length increased by 15% relative to the wild type in the presence of salt is shown (Fig. 12, A and B). Transcript accumulation of \( EsCBL10a \) and \( EsSOS3 \) was similar in the single and double lines (Fig. 12C). These results suggest that \( EsCBL10a \) and \( EsSOS3 \) are sufficient to confer salt tolerance and increase growth in the presence of salt above that of wild-type Arabidopsis.

DISCUSSION

The Duplication of the Eutrema CBL10 Calcium Sensor Increased the Complexity of Calcium-Mediated Signaling and Confers Salt Tolerance

Duplication of genes has been shown to underlie plant adaptation by expanding signaling responses during growth in adverse environments. For example, gene duplication has been shown to contribute to adaptation to cooler temperatures, tolerance to heavy-metal-polluted soils, and expansion of defense responses (Hanikenne et al., 2008; Gonzales-Vigil et al., 2011; Ilyas et al., 2015; Simon et al., 2015). The duplication of \( CBL10 \) in Eutrema provided the opportunity to determine if increasing the number and expanding the activities of a calcium sensor confer an adaptive benefit during plant growth in saline soils.

To determine the contribution of \( EsCBL10a \) and \( EsCBL10b \) to calcium-mediated signaling in response to salt, the effects of down-regulating their expression...
Figure 10. The hydrophobic domain of EsCBL10a is important for complementation of Atsos3. Chimeric EsCBL10a (E10a)/ EsCBL10b (E10b) proteins were expressed in Atsos3 and growth in the absence (Control) and presence of salt (75 mM NaCl) was monitored. A, Schematic representation of the chimeric proteins. Red, E10a protein; blue, E10b protein; I, insertion of seven amino acids in E10a; HD, hydrophobic domain; V, variable sequence of amino acids; Ca, calcium-binding domains; S, Ser phosphorylation site. B, Photographs of wild type (WT), Atsos3, and Atsos3 expressing E10a, E10b, or the chimeric proteins. Bar = 1 cm for all images. C, Fresh weight and length of the primary root were measured to quantify growth. Data are means ± se of
were measured and their protein activities were examined. Both EsCBL10a and EsCBL10b function in the response of Eutrema to salt and likely have distinct roles (Fig. 3). Both genes complemented Atcbl10, demonstrating functional conservation between CBL10 in Arabidopsis and Eutrema (Fig. 4). However, evidence for a divergence in protein activity was seen in the different abilities of EsCBL10a and EsCBL10b to activate the SOS pathway and complement Atsos3 (Figs. 5, 7, and 13). EsCBL10a complemented Atsos3, suggesting that it might share overlapping functions with EsSOS3 (Fig. 7). However, EsCBL10a and EsSOS3 complement Atsos3 in an organ-specific manner and have differing abilities to complement Atcbl10 and activate the SOS pathway, suggesting that these genes have distinct roles in response to salt (Figs. 4, 7, and 13; Supplemental Figs. S10 and S11). Our results demonstrate that, as a result of the duplication of CBL10, Eutrema has three calcium sensors, EsCBL10a, EsCBL10b, and EsSOS3, with both overlapping and distinct functions involving multiple signaling pathways (Fig. 13).

Because the duplication in Eutrema expanded the complexity of calcium sensors that contribute to its salt tolerance, and because Arabidopsis is more salt-sensitive and contains only one CBL10 gene, we asked if the combined action of more than one Eutrema calcium sensor could confer salt tolerance to Arabidopsis. Even though EsCBL10a and EsCBL10b are necessary for Eutrema’s salt tolerance (Fig. 3), expression of EsCBL10a and EsCBL10b in combination in Atcbl10 was not consistently sufficient to confer increased salt tolerance to Arabidopsis. When EsCBL10a and EsSOS3 were expressed together in Atsos3, root growth increased beyond that of the wild type and Atsos3 expressing the individual genes, suggesting that the combined functions of EsCBL10a and EsSOS3 are important for root growth in the presence of salt and can confer salt tolerance to Arabidopsis (Fig. 12). Because the components of the EsCBL10 signaling pathways are likely limiting in Arabidopsis, future studies in which these components are identified and expressed in Arabidopsis might be necessary for increasing salt tolerance.

**Multiple Mechanisms Underlie Differences in EsCBL10 Gene Function**

Differences in CBL10 function can be achieved through changes in protein localization, affinity for calcium, interaction with and activation of a target protein, or phosphorylation by a target protein. We reasoned that the enhanced ability of EsCBL10b to activate the SOS pathway might be due to a stronger interaction with SOS2 and tested this hypothesis using yeast two-hybrid assays. EsCBL10b interacted with AtSOS2 more strongly than EsCBL10a, but at a level similar to AtCBL10 (Fig. 6). This result suggests that interaction alone cannot explain pathway activation and that additional factors must play a role.

Experiments with chimeric proteins revealed that the ability of EsCBL10b to strongly activate the SOS pathway resides in the first eight amino acids of the protein (Fig. 9). The AtCBL10 hydrophobic domain is large enough to span the membrane (Quan et al., 2007); however, this would mean that the amino acids that underlie EsCBL10b’s activation of the SOS pathway would be extracellular. If the EsCBL10b hydrophobic domain associates with the membrane via a hydrophobic loop (Winter et al., 2009; Hernández-Gras and Boronat, 2015; Xu et al., 2016), the amino acids 5’ to the hydrophobic domain would reside in the cytosol and might influence subcellular localization and/or interaction with a target protein. In EsCBL10a, seven amino acids are present in this region, suggesting that the reduced ability of EsCBL10a to activate the SOS pathway might be due to this insertion (Fig. 9).

Because EsCBL10a only weakly activated the SOS pathway and showed little interaction with SOS2, yeast two-hybrid assays were performed to determine if interaction with a different kinase underlies EsCBL10a’s ability to complement Atsos3. Four CIPK proteins were identified that interact with EsCBL10a but not EsCBL10b or AtCBL10 (Fig. 8). Three of the four also interact with AtSOS3, suggesting that EsCBL10a might be performing both SOS3-like and SOS3-independent functions (Fig. 8). Two of the identified CIPK proteins, AtCIPK16 and AtCIPK6, have known roles in salt tolerance (Tsou et al., 2012; Roy et al., 2013); both have been shown to interact with and activate the potassium transporter AtAKT1 (Lee et al., 2007). In addition, AtCIPK6 has been shown to interact with AtSOS3 to recruit the potassium transporter AtAKT2 to the plasma membrane (Held et al., 2011). These results suggest that EsCBL10a could interact with AtCIPK6 and/or AtCIPK16 proteins to regulate potassium levels in Atsos3, thereby alleviating some of the toxic effects of sodium. A homolog of AtCIPK16 has been identified in Eutrema but the role of EsCIPK16 in plant responses to salt has not been explored (Amarasinghe et al., 2016). Future studies focusing on EsCBL10a interaction with EsCIPK proteins and down-regulation of EsCIPK gene expression will link the associated proteins to salt tolerance.
tolerance. EsCBL10a also interacted with AtCIPK13 and AtCIPK18 (Fig. 8); however, a role for these genes in salt tolerance has not been reported. Single and double mutant phenotypes (based on the phylogenetic relatedness of the two genes) were assessed; however, salt-sensitive phenotypes were not detected (data not shown). These results suggest that these genes do not function with EsCBL10a to complement Atsos3 or that additional redundant genes prevent the manifestation of a salt-sensitive phenotype.

EsCBL10 chimeric proteins revealed that the EsCBL10a-specific function resides in the amino terminus within the hydrophobic domain. While little is known about the function of the hydrophobic domain in EsCBL10, studies in Arabidopsis and poplar (Populus trichocarpa) suggest that the CBL10 hydrophobic domain is important for subcellular localization. Removal of the domain from AtCBL10 prevented it from recruiting AtSOS2 to the plasma membrane (Quan et al., 2007), while its removal from the duplicated CBL10 proteins in poplar prevented their localization to the vacuolar membrane (Tang et al., 2014). When compared with AtCBL10 and EsCBL10b, two differences in amino acid sequence were observed in the hydrophobic domain of EsCBL10a (major element; Fig. 10). A second element was identified in EsCBL10a that confers partial function (minor element; Fig. 10). The chimeric protein containing the EsCBL10b hydrophobic domain and the EsCBL10a variable domain was able to restore rosette growth to wild-type-like levels but not root length (bN2; Fig. 10). Because of its proximity to the hydrophobic domain, this region might influence localization of the protein. Alternatively, while the three-dimensional structure of other CBL proteins indicates that this region would be unlikely to directly interact with a protein partner, it might regulate initial recognition of that protein. Future studies will involve localization of tagged EsCBL10 proteins driven by their native promoters in Eutrema. However, the low abundance of the CBL10 protein when expressed from the native promoter and its uncertain localization using a constitutive promoter (Kim et al., 2007; Quan et al., 2007; Tang et al., 2014) suggest that these experiments might prove to be difficult.

Changes in CBL10 Expression May Have Also Increased the Complexity of Calcium-Mediated Signaling in Eutrema’s Response to Salt

Our results indicate that the expression patterns of the EsCBL10 genes diverged; the EsCBL10b transcript is...
predominantly present in aerial tissues while the EsCBL10a transcript is present throughout the plant (Fig. 2). Alternative splicing of AtCBL10 results in two major variants, only one of which encodes a functional protein (Quan et al., 2007). Most of the EsCBL10a and EsCBL10b splice variants identified likely encode nonfunctional proteins due to premature stop codons or the absence of domains required for binding calcium (Supplemental Figure 12. EsCBL10a and EsSOS3 in combination confer salt tolerance. Atsos3 plants expressing EsCBL10a and EsSOS3 individually were crossed to generate heterozygous plants expressing both genes and growth in the absence (Control) or presence of salt (75 mM NaCl) was monitored. A, Photographs of wild type (WT), Atsos3, and Atsos3 expressing EsCBL10a (E10a), EsSOS3 (E3), or both genes (a3D). Bar = 1 cm for all images. B, Fresh weight and the length of the primary root were measured to quantify growth. Data are means ± se of at least 24 seedlings per genotype grown in three independent experiments. For all graphs, different letters indicate significant differences between genotypes (two-way ANOVA, Tukey-Kramer HSD, \( P \leq 0.05 \)). C, Transcript accumulation. ACTIN, Loading control. One representative image of three replicates is shown.

Figure 13. The duplication of EsCBL10 increased the complexity of signaling in response to salt. In Arabidopsis, AtSOS3 and AtCBL10 interact with and activate the protein kinase AtSOS2, which, in turn, activates the sodium-proton exchanger AtSOS1 (AtSOS pathway) in the plasma membrane (PM). AtSOS1 then transports sodium out of the cell, preventing its toxic accumulation. AtSOS3 and AtCBL10 have additional functions outside of activation of the AtSOS pathway. Homologs were identified in Eutrema and one gene, EsCBL10a, has been duplicated. EsCBL10b has an enhanced ability to activate the SOS pathway and complements the Atcbl10 salt-sensitive phenotype. EsCBL10a complements both the Atcbl10 and Atsos3 salt-sensitive phenotypes but only weakly activates the SOS pathway. EsSOS3 activates the SOS pathway and complements the Atsos3 salt-sensitive phenotype.
Evolutionary Models May Explain Differences in Eutrema CBL10 Function

Several models have been developed to describe how genes diverge in function so that both are maintained (Flagel and Wendel, 2009; Hahn, 2009; Innan and Kondrashov, 2010; Kondrashov, 2012). Of these, two might best explain how EsCBL10a and EsCBL10b function diverged. Neofunctionalization postulates that, following a gene duplication, one gene maintains the original function, while the other gene, free from selective constraints, develops a novel, advantageous function (Ohno, 1970). In the case of the EsCBL10 genes, EsCBL10b might have maintained the original function (activation of the SOS pathway) while EsCBL10a developed a novel function (complementation of Atsos3). Subfunctionalization (duplication-degeneration-complementation) postulates that the original gene (before the duplication) had multiple functions that were later partitioned between the duplicated genes (Force et al., 1999). In the case of the EsCBL10 genes, EsCBL10b (before the duplication) would have had both functions (activation of the SOS pathway and complementation of Atsos3). Following the duplication, one function was maintained in EsCBL10b (activation of the SOS pathway) and one function (complementation of Atsos3) was lost. Similarly, EsCBL10a maintained one function (complementation of Atsos3) and lost the other function (activation of the SOS pathway). Once the functions were partitioned, each gene could acquire changes that might enhance its function; something that might not have been possible when one gene performed both functions (escape from adaptive conflict; Hughes, 1994; Des Marais and Rausher, 2008). To distinguish between these models and determine if EsCBL10a developed the function that complements Atsos3 before the duplication (subfunctionalization) or after the duplication (neofunctionalization), functional data for CBL10 from additional species will be required.
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(http://wmd3.weigelworld.org; Schwab et al., 2006; Oosowski et al., 2008). Two 21-bp sequences per target (EsCBL10a, EsCBL10b, and EsCBL10a/EsCBL10b) were identified. Primers (Supplemental Table S2) containing the amiRNA sequences were generated and the amiRNA sequences was incorporated into the AtMIR319a precursor gene located on the pRS300 vector (Dr. Detlef Weigel, Max Planck Institute for Developmental Biology; Oosowski et al., 2008). The AtMIR319a gene containing the desired amiRNA sequence was digested with EcoRI and XhoI and subcloned into the corresponding site of the plant binary vector pEZT-NL (Nrs. Sean Cutler and David W. Ehrhardt, Carnegie Institution of Washington) downstream of the CaMV 35S promoter. 

Agrobacterium tumefaciens EH105 containing the binary vector was used to transform Eutrema via the floral dip method (Clough and Bent, 1998). T1 seed was germinated on soil for 1.5 weeks and then sprayed three times with Basta (100 mg/L, glufosinate ammonium; Rently Herbicide; Bayer Crop Science) at 3-4 intervals. T1 lines with antibiotic resistance were subsequently transferred to pots and grown to collect T2 seed. Single insertion lines were identified by screening T2 seed on EBM plates containing 7.5 mg L\(^{-1}\) glufosinate ammonium (Santa Cruz Biotechnology) and selecting lines with 75% resistance. Homozygous lines were identified by screening T3 seed on EBM plates with glufosinate ammonium and selecting lines with 100% resistance. Salt assays were performed as described above.

The efficiency of amiRNA targeting was determined using reverse transcription quantitative PCR. RNA was isolated from 3-week-old seedlings using Trizol (Invitrogen). RNA was treated with RQ1 DNase (Promega), purified using the RNeasy Plant Mini Kit (Qiagen), and used to synthesize cDNA (M-MLV Reverse Transcriptase; Promega). Reverse transcription quantitative PCR was performed using the LightCycler Fast Start DNA MasterSYBR Green 1 Kit (Roche) in the LightCycler 1.5 instrument (Roche). Melting curve analysis was performed to verify that a single PCR product was amplified. Ct values were calculated using the LightCycler software 4.0 package (Roche). The relative fold change in expression was determined using the calculation 2\(^{-DDCT}\) (Schmittgen and Livak, 2008). All primers are provided in Supplemental Table S1.

**Complementation Assays**

The functions of EsCBL10a, EsCBL10b, and EsSOS3 were analyzed by expressing each gene in the Atcbl10 and Atsos3 mutants. Full-length coding sequences without stop codons were amplified from cDNA and cloned into pGEM-T Easy (Promega). All PCR amplifications were performed using Phusion High-Fidelity DNA Polymerase (ThermoFisher Scientific); primers are provided in Supplemental Table S1. A. tumefaciens EHA105 was used to transform Eutrema calcium sensor confers salt tolerance to Arabidopsis, plants expressing the corresponding site of the CaMV 35S promoter. A. tumefaciens LBA4404 containing the binary vector was used to transform Atcbl10 and Atsos3 via the floral dip method (Clough and Bent, 1998). A similar strategy was used to generate single insertion, homozygous lines as described above for the amiRNA lines, except MS medium was used in place of EBM. A. tumefaciens containing the CaMV promoter was transformed into Dam/Cbl10 and Atsos3 downstream of the CaMV 35S promoter were provided by Dr. Yan Guo (China Agricultural University). To determine if coexpression of more than one gene was expressed with the Arabidopsis, plants expressing the corresponding site of the CaMV 35S promoter were selected on synthetic dropout medium lacking uracil and Trp (Clontech/TaKaRa) containing 1 µM KCl with the designated concentrations of NaCl and cultured at 30°C for 4 d.

To monitor the level of CBL10 expression in AXT3K, spheroplasts were generated by removing the cell wall with Lyticase (Sigma; Yilmaz et al., 2012). RNA was extracted from spheroplasts using the TRI REAGENT (Invitrogen; Chomczynski and Sacchi, 1987, 2006; Yilmaz et al., 2012), treated with TURBO DNase (ThermoFisher Scientific), and purified using the RNeasy MiniElute Cleanup Kit (Qiagen). cDNA was synthesized using SuperScript III Reverse Transcriptase (Life Technologies) and RNA was removed using RNaseH (Life Technologies). All PCR reactions were performed using Recombinant Taq Polymerase (Invitrogen); primers are provided in Supplemental Table S1.

**Yeast Two-Hybrid Screens**

EsCBL1a-interacting kinases were identified using yeast two-hybrid screens. Each gene to be tested for yeast two-hybrid interaction was amplified by PCR using Phusion High-Fidelity DNA Polymerase (ThermoFisher Scientific); primers are provided in Supplemental Table S1. For CIPK genes 1, 2, 4, 6, 7, 9 to 11, 13, 18, 19, 21, and 24 (SOS2), the PCR products were digested with EcoRI and BamHI and cloned into the corresponding site of the pGADT7 and pGBKTK7 vectors (Clontech/TaKaRa), which allow for expression of the genes as fusion proteins with the GAL4 DNA-activation domain or the GAL4 DNA-binding domain, respectively. For CIPK genes 5, 8, 12, 14 to 17, 20, 22, and 26, the PCR products were digested with XmaI and XhoI and cloned into the corresponding site of pGADT7, pGBKTK7 does not have a XhoI site but the SacI site produces the same overhang, so each CIPK gene digested with XmaI and XhoI was cloned into pGBKTK7 digested with XmaI and SacI. For CIPK25, which contains an internal Xhol site, the PCR product was digested with XmaI and Sall and cloned into the corresponding site of pGBKTK7 digested with XmaI and XhoI. The pGADTK7 clones were transformed into S. cerevisiae strain Y2HGold (Clontech/TaKaRa) except for the AtSOS3 clone, which had been previously transformed into S. cerevisiae strain AH109. The pGBKTK7 clones were transformed into S. cerevisiae strain Y187 (Clontech/TaKaRa). Yeast were mated and grown on SD medium minus Leu and Trp (Clontech/TaKaRa) to select for diploid yeast expressing both constructs. To determine interaction, serial dilutional yeasts were colonies were grown on SD medium minus Leu and Trp and without His and adenine or with the addition of 0.5 mM 3-amino-1,2,4-triazole (Sigma) and incubated for 5 d.

**Chimeric Proteins**

To generate chimeric EsCBL10 for expression in yeast, EsCBL10a and EsCBL10b were cloned into pDR195 (cloning described in “Yeast Salt Assays” above). For the first set of chimeric proteins, HindIII was used to generate two sites in the plasmid, one in the vector and the other within each gene. The resulting fragments containing the amino terminus of each gene were then cloned into the vector containing the carboxy terminus of the other gene. The plasmids were digested with AgeI and NotI and a fragment containing the chimeric gene was subcloned into the corresponding site of the pFL32T plasmid to be expressed with AtSOS2 in AXT3K. For the second set of chimeric proteins, each gene fragment (aN2, aC2, bN2, and bC2) was PCR amplified using Phusion High-Fidelity DNA Polymerase (ThermoFisher Scientific); primers are provided in Supplemental Table S2. The plasmids were designed so that the aN2 and bC2 and the bN2 and aC2 fragments would overlap. The fragments were amplified by PCR and the full-length chimeric gene cloned into pGEM-T Easy (Promega). The plasmid was then digested with XhoI and NotI and a fragment containing the chimeric gene was subcloned into the corresponding site of the pDR195 vector. The resulting plasmid was digested with Agel and NotI and the fragment containing the chimeric gene was subcloned into the corresponding site of the pFL32T plasmid to be expressed with AtSOS2 in AXT3K. For the third set of chimeric proteins, EsCBL10a and EsCBL10b cloned into pDR195 were transformed into dam–/dcm– competent cells (New England Biolabs) to get a methylation-free plasmid. The resulting plasmids were digested with Agel and XhoI and the fragments containing the amino terminus of one gene were cloned into the plasmid containing the carboxy terminus of the other gene. The
resulting plasmid was digested with AgeI and NotI and the fragment containing the chimeric gene was subcloned into the corresponding site of the pFL32T plasmid to be expressed with AtSOS2 in AXT3K.

For expression in Arabidopsis, each chimeric gene was amplified from the yeast plasmids and cloned into pGEM-T Easy (Promega). All PCR amplifications were performed using Thunson High-Fidelity DNA Polymerase (ThermoFisher Scientific); primers are provided in Supplemental Table S1. Each chimeric gene was digested with XhoI and BamHI and subcloned into the corresponding site of the plant binary vector pEZT-NL containing the CaMV 35S promoter. Transformation of Arabidopsis and generation of homozygous lines were performed as described above in “Complementation Assays.”

Statistical Analysis

To determine significant differences in growth, experiments were organized and analyzed as a randomized complete block design with genotypes and salt concentrations as treatments and individual experiments as replicates. Treatment effects were assessed using a full-factorial mixed-model ANOVA in JMP, Version 11 (SAS Institute; 1989-2007). In these analyses, treatments were considered fixed effects and replicates random effects. The normality of the distributions of all dependent variables was analyzed by examining a plot of the residuals from a full-factorial ANOVA of untransformed data. A Shapiro-Wilk test (Shapiro and Wilk, 1965) was performed to assess normality, and Bartlett’s (Bartlett, 1937) and Levene’s (Levene, 1960) tests were performed to evaluate the homogeneity of variance. Based on the pattern of distribution and the results of these tests, a nonparametric approach was used to analyze the data. Data were rank transformed using Microsoft Excel (function: RANK) followed by an ANOVA and Tukey’s HSD test for multiple comparisons of means (Conover and Iman, 1981). The HSD values from rank-based ANOVA were then applied to the actual means for each measurement (i.e. not the ranks used in ANOVA). Statistical significance was assigned at \( p \leq 0.05 \), and all tests of significance were two sided.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AtMgZ300020 (AtCBL10), AtEG24270 (AtSOS3), ESQ53857 (EsCBL10a, Thalhiv1002619m), ESQ58334 (EsCBL10b, Thalhiv1002809m), and ESQ31966 (EsCOS3, Thalhiv1004891m).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. AtCBL10, EsCBL10a, and EsCBL10b are alternatively spliced.

Supplemental Figure S2. AmiRNA annealing sites and specificity

Supplemental Figure S3. Reduced expression of EsCBL10a and EsCBL10b results in decreased root growth.

Supplemental Figure S4. EsCBL10a and EsCBL10b complement the Atch10 fertilization defect.

Supplemental Figure S5. EsCBL10b interaction with AtSOS2 is orientation specific.

Supplemental Figure S6. Orientation of the yeast two-hybrid affects CBL10 interaction with the CIPK proteins.

Supplemental Figure S7. Chimeric EsCBL10 proteins.

Supplemental Figure S8. The chimeric EsCBL10 genes are expressed in the Atch10 and Atsos3 mutants.

Supplemental Figure S9. All chimeric proteins complement the Atch10 salt-sensitive phenotype.

Supplemental Figure S10. EsSOS3 activates the Arabidopsis and Eutrema SOS pathways.

Supplemental Figure S11. EsSOS3 does not complement the Atch10 salt-sensitive phenotype.

Supplemental Table S1. Primers.

Supplemental Table S2. Primers used for generating the amiRNA constructs.

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LITERATURE CITED


Hughes AL (1960) Robust tests for equality of variances. Technometrics 2: 135–143


