

Arabidopsis CALCINEURIN B-LIKE10 Functions Independently of the SOS Pathway during Reproductive Development in Saline Conditions^{1[OPEN]}

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The accumulation of sodium in soil (saline conditions) negatively affects plant growth and development. The Salt Overly Sensitive (SOS) pathway in *Arabidopsis thaliana* functions to remove sodium from the cytosol during vegetative development preventing its accumulation to toxic levels. In this pathway, the SOS3 and CALCINEURIN B-LIKE10 (CBL10) calcium sensors interact with the SOS2 protein kinase to activate sodium/proton exchange at the plasma membrane (SOS1) or vacuolar membrane. To determine if the same pathway functions during reproductive development in response to salt, fertility was analyzed in wild type and the SOS pathway mutants grown in saline conditions. In response to salt, CBL10 functions early in reproductive development before fertilization, while SOS1 functions mostly after fertilization when seed development begins. Neither SOS2 nor SOS3 function in reproductive development in response to salt. Loss of CBL10 function resulted in reduced anther dehiscence, shortened stamen filaments, and aborted pollen development. In addition, *cb10* mutant pistils could not sustain the growth of wild-type pollen tubes. These results suggest that CBL10 is critical for reproductive development in the presence of salt and that it functions in different pathways during vegetative and reproductive development.

Because the seed is the source of food and feed in many major agronomic crops, successful reproductive development underlies the economic importance of many crop species. Crop yields are often well below reported record yields, largely due to growth in unfavorable environmental conditions that include too much or too little soil moisture, temperature extremes, and nutrient or other element toxicities and deficiencies (Boyer, 1982; Cramer et al., 2011). Therefore, understanding the mechanisms by which plants modify their reproductive development in unfavorable environmental conditions will be critical for developing methods to maintain and improve crop productivity.

Accumulation of salts in the soil solution (saline conditions) affects more than 400 million hectares, over 6% of the world's land mass (FAO, 2002). Estimates

suggest that more than 12 million hectares of irrigated land has gone out of production as a result of salinization (Nelson and Mareida, 2001), that more than 50% of irrigated land is salt-affected in some countries (FAO, Aquasat Database), and that at least 3 hectares of arable land are lost worldwide each minute due to salinity. In angiosperms, reproductive development is often inhibited in saline conditions (Babu et al., 2012; Maas et al., 1986; Munns and Rawson, 1999; Rubio et al., 2009; Samineni et al., 2011). For example, in rice, panicle initiation (the beginning of the reproductive phase) is more sensitive to salt (NaCl) than all other developmental stages except for early seedling growth (Asch and Wopereis, 2001; Khatun and Flowers, 1995). In saline conditions, reduced pollen viability and abnormal pistil development resulted in reduced spikelet (floral unit) number, fertility, and yield (Asch and Wopereis, 2001; Khatun and Flowers, 1995). In *Arabidopsis thaliana*, addition of 200 mM NaCl to hydroponic medium reduced fertility (seed set) in newly formed siliques by 90% compared with seed set in control plants (Sun et al., 2004). This reduction in fertility was associated with decreased stamen elongation, collapse of pollen grains, ovule abortion (indicated by the presence of callose in the ovules), and disrupted divisions within the female gametophyte (Sun et al., 2004).

As sessile organisms, plants have highly complex signaling pathways that enable them to respond to and modify their growth in diverse environments. Forward genetic screens in *Arabidopsis* have identified mutants with increased salt sensitivity relative to wild type. Cloning of the associated genes and characterization of

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their protein products led to the identification of the Salt Overly Sensitive (SOS) pathway that functions during vegetative development to remove sodium from the cytosol, preventing its accumulation to toxic levels. In this pathway, accumulation of sodium triggers an influx in cytosolic calcium. Increased calcium is perceived by two calcium-binding proteins, SOS3 (roots) and CALCINEURIN B-LIKE10 (CBL10, also known as SOS3-LIKE CALCIUM-BINDING PROTEIN8, leaves), that interact with and activate the SOS2 serine/threonine protein kinase (Halfter et al., 2000; Kim et al., 2007; Liu et al., 2000; Liu and Zhu, 1998; Quan et al., 2007). SOS2 phosphorylates SOS1, a sodium/proton exchanger, initiating transport of sodium out of the cell (Lin et al., 2009; Qiu et al., 2002, 2003; Quintero et al., 2002; Shi et al., 2000) or activates an unknown transporter on the vacuolar membrane, leading to sequestration of sodium in the vacuole (Qiu et al., 2004).

The goal of this research was to identify the mechanisms by which plants modify reproductive development in response to NaCl. As a first step, we determined if the same genes function during vegetative and reproductive development by comparing fertility in wild-type *Arabidopsis* and the SOS pathway mutants. We found that CBL10 functions independently of the SOS pathway during reproductive development. Based on characterization of the *cbl10* mutant phenotype when plants were grown in the presence of salt, we

found that CBL10 is important for both stamen and pistil function.

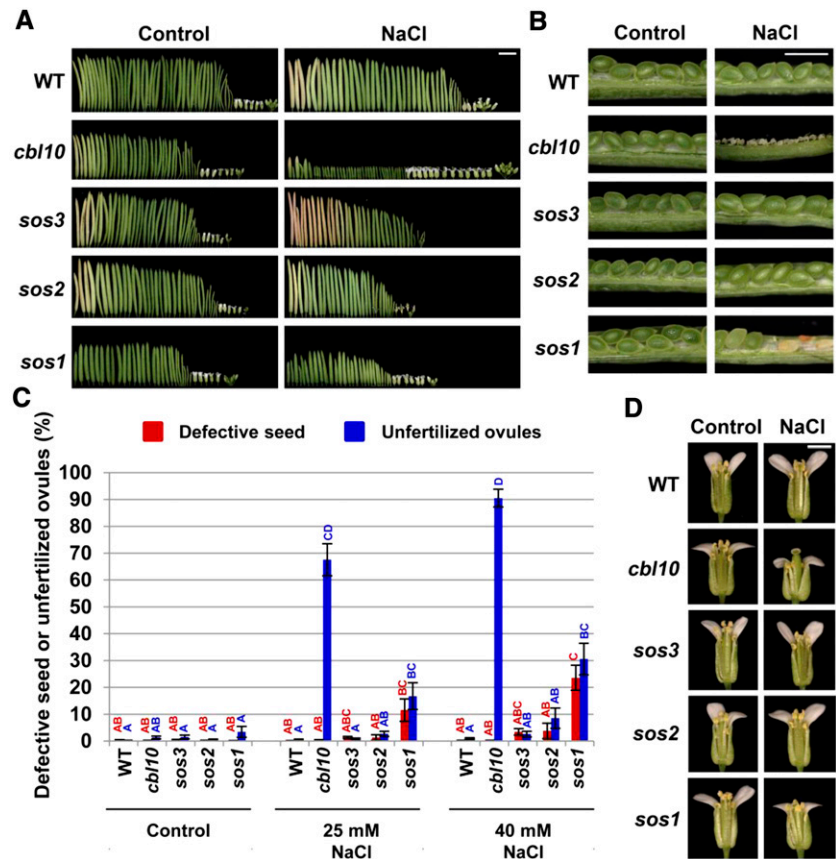
RESULTS

Fertility Is Reduced in the *cbl10* Mutant in Response to Salt

To identify genes critical for reproductive development in saline conditions, wild-type and SOS pathway mutant plants were grown in the absence of salt for 3 weeks and then, at the start of inflorescence development, left untreated (control) or treated with salt (NaCl). After 3 weeks of treatment, inflorescence, flower, silique, and seed development were evaluated. In the presence of salt, *sos2* and *sos3* siliques were similar in length to those in wild type, while siliques from the *cbl10* and *sos1* mutants were significantly shorter than wild-type, *sos2*, and *sos3* siliques (Supplemental Fig. S1A; Fig. 1A). The number of seed positions (an indicator of the total number of ovules that developed in pistils) was monitored within representative siliques. The total number was the same in all siliques in control conditions and remained unchanged with salt treatment for all siliques except those from the *sos1* mutant in which total seed positions were reduced significantly at both salt concentrations (Supplemental Fig. S1B).

Seed development in *cbl10* and *sos1* siliques was the same as in wild type when plants were grown in control

Figure 1. CBL10 functions in reproductive development independently of the SOS pathway when plants are treated with salt. Reproductive development was examined in wild-type (WT) and mutant plants left untreated (Control) or treated with salt (40 mM NaCl, unless otherwise indicated) for 3 weeks. A, Siliques from a primary inflorescence (oldest to youngest, left to right). Bar (5 mm, top right) shows magnification for all images. B, One 10-d-old silique per plant that developed after 2 weeks of treatment was opened to monitor seed set. Bar (1 mm, top right) shows magnification for all images. C, The number of defective seed and unfertilized ovules per silique was graphed as a percent of the total number of positions in a silique. Siliques were examined in five independent experiments (mean: 32 siliques; range: 28–36). Defective seed, seed containing a white or brown aborted embryo; Unfertilized ovules, ovules that were not fertilized or seed that aborted just after fertilization. The data were rank transformed and an ANOVA conducted using a mixed model. Any pair of genotypes/treatments that do not share the same letter are significantly different ($P \leq 0.05$) based on a Tukey HSD significance test. Bars represent se. D, Stage 14 flowers (Smyth et al., 1990) with one or two sepals and petals removed. Bar (1 mm, top right) shows magnification for all images.



conditions; however, in response to salt treatments, there was a significant increase in unfertilized ovules in the *cb110* mutant and in both unfertilized ovules and defective seed in the *sos1* mutant (Fig. 1, B and C). The increase in unfertilized ovules suggested that *CBL10* and *SOS1* might function during floral organ development, so stage 14 flowers (Smyth et al., 1990) from wild type and the mutants were examined. When plants were treated with salt, stamens in *cb110* flowers did not elongate above the pistil, while in *sos1* stamens elongated to release pollen onto the stigma similar to what is observed in wild-type flowers (Fig. 1D).

To determine if the changes in reproductive development in the salt-treated *cb110* and *sos1* mutants represent an indirect effect of reduced vegetative development, plant growth was assessed by measuring rosette fresh weight at the conclusion of the salt treatments. When plants were treated with salt at the start of inflorescence development, growth of *sos1* was reduced, while growth of the *cb110* mutant was the same as in wild type (Supplemental Fig. S1C), indicating that the reproductive phenotype in the *cb110* mutant is not due to defects in vegetative development. Additional evidence for *CBL10*'s role in flower development or function included the absence of a salt effect on inflorescence height (Supplemental Fig. S1D) or number of siliques (Supplemental Fig. S1E) in the *cb110* mutant.

To verify that the sterile phenotype observed in the *cb110* mutant is due to a mutation in the *CBL10* gene, *CBL10* was overexpressed in the *cb110* mutant. *CBL10* restored fertility to wild-type levels (Supplemental Fig. S2), indicating that the phenotype is linked to the *CBL10* gene.

CBL10 Is Expressed in Flowers

To determine the pattern of *CBL10* expression during flower development, we analyzed both its mRNA accumulation and promoter activity using reverse transcriptase PCR and *CBL10pro:GUS* fusions, respectively. Both *CBL10* (bottom band) and an alternatively spliced variant (top band) were present throughout flower development (Fig. 2A). Analysis of 18 independent *CBL10pro:GUS* transgenic lines showed expression in stamen filaments (18 of 18), and the style (18 of 18) and transmitting tract (14 of 18) of the pistil (Fig. 2B).

The *cb110* Mutant Exhibits Multiple Defects in Floral Organ Function in Response to Salt

During flower development, *Arabidopsis* flowers open in the morning and close in the afternoon (van Doorn and Van Meeteren, 2003). This cycle continues for several days until silique development begins and flowers senesce. Flower opening and closing were examined in stage 14 (Smyth et al., 1990) wild-type and *cb110* flowers. In the untreated *cb110* mutant, flower opening and closing were similar to what was observed in wild type. In the salt-treated *cb110* mutant, flowers

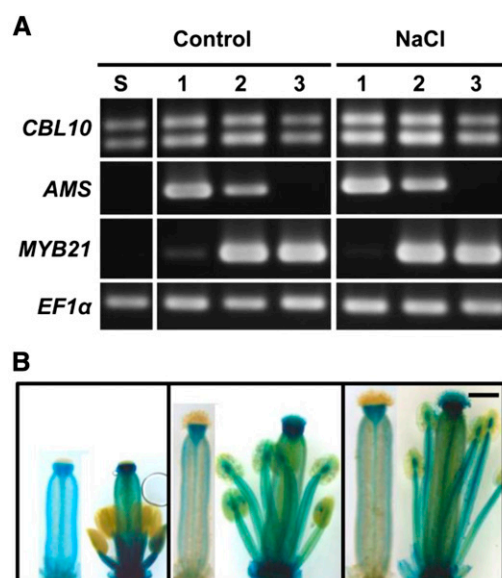


Figure 2. *CBL10* is present in stamens and pistils throughout flower development. A, RNA was isolated from wild-type flowers collected in three pools representing different stages of development (Smyth et al., 1990) from plants left untreated (Control) or treated with salt (40 mM NaCl). 1, Meristem formation and early flower development (stages 1–11); 2, anthesis (stages 12 and 13); 3, fertilization (stages 14 and 15). *AMS*, Marker for early flower development; *MYB21*, marker for late flower development; *EF1α*, loading control. RNA was also isolated from untreated seedlings (S) as a control. For the *CBL10* reaction, bottom band, *CBL10*; top band, alternatively spliced transcript. B, Expression patterns of the *GUS* reporter gene under the control of the *CBL10* promoter were examined in wild-type flowers at different stages of development (stages 12, 13, and 15, left to right; Smyth et al., 1990). One representative line of 18 total lines is shown. Bar (1 mm, right) shows magnification for all images.

did not close in the afternoon but closed during senescence (Supplemental Fig. S3).

In wild-type flowers at the time of anthesis and fertilization (stages 12–14), anthers dehisce to release pollen, stamens elongate to position the anther above the pistil, and petals expand to open the flower (Smyth et al., 1990). These processes were impaired in the *cb110* mutant treated with salt. Based on the severity of the phenotype, *cb110* flowers were categorized into three phenotypes. Phenotype 1 flowers exhibited the most severe phenotype; anthers did not dehisce, stamens did not elongate, and petals did not expand (Fig. 3, A and B; Supplemental Fig. S4A). In phenotype 2 flowers, petals expanded, but stamens only elongated partially and anthers did not dehisce (Fig. 3, A and B; Supplemental Fig. S4A). Phenotype 3 flowers exhibited the least severe phenotype; most anthers dehisced, petals expanded, and stamens elongated, but not as fully as in wild type (Fig. 3, A and B; Supplemental Fig. S4A). Often the first flowers to develop on an inflorescence were phenotypes 2 and 3. Over time, phenotype 1 appeared more often, likely due to the buildup of salt in the inflorescence giving rise to the more severe phenotype 1 flowers.

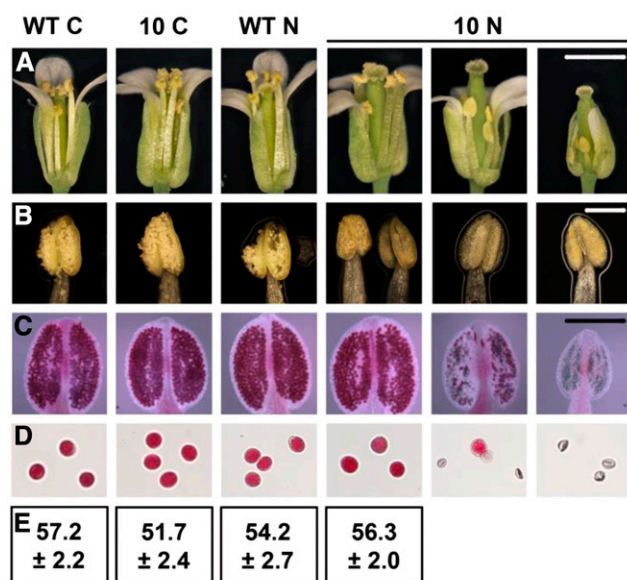


Figure 3. Anther dehiscence is reduced, stamen elongation is decreased, and pollen development is aborted in the salt-treated *cbl10* mutant. A, Flowers at stage 14 (Smyth et al., 1990) from wild-type (WT) and *cbl10* (10) plants left untreated (control, C) or treated with salt (40 mM NaCl, N) were photographed. Three *cbl10* stamen phenotypes were observed for the salt-treated *cbl10* plants. Phenotype 3, left; phenotype 2, middle; phenotype 1, right. Bar (1 mm, top right) shows magnification for all images. B, Representative anthers from stage 14 flowers (Smyth et al., 1990) were photographed. Bar (0.25 mm, top right) shows magnification for all images. C, Anthers from flowers at stage 12 (Smyth et al., 1990) were incubated in Alexander's stain, which stains viable pollen red and nonviable pollen blue (Peterson et al., 2010). Bar (0.25 mm, top right) shows magnification for all images. D, Pollen was released from anthers stained with Alexander's stain and photographed. E, Pollen from each plant was used to pollinate pistils of wild-type plants grown in the absence of salt. The number of seed per silique was counted 10 d after pollination. The mean number of seed and the SE are shown for 23 siliques per genotype per treatment from three independent experiments.

To determine if pollen development was affected in the salt-treated *cbl10* mutant, pollen viability was examined using Alexander's stain, and fertilization was examined by pollinating wild-type pistils. Pollen from phenotype 1 and 2 flowers collapsed and was not viable (Fig. 3, C–E). The anthers from these flowers did not dehisce, and, when anthers were opened manually, little or no pollen could be obtained to assess fertilization by crossing to wild-type plants. Pollen from phenotype 3 flowers was viable and able to successfully fertilize ovules within a wild-type pistil (Fig. 3, C–E). To separate CBL10's role in stamen function from a possible role in male gametophyte development, heterozygous *CBL10*^{−/−} mutant plants were allowed to self-pollinate or outcrossed to wild-type plants as pollen donors to analyze transmission of the mutant allele in progeny seedlings. Seed from self-pollinated plants (untreated and salt-treated) and from crosses (pollen from salt-treated plants used to pollinate pistils from untreated plants) was collected and seedlings genotyped

to determine segregation of the mutant allele. The observed ratio of wild-type to mutant allele was not significantly different from the expected ratio for any combination (Supplemental Table S1), indicating that the *CBL10* gene does not have a role in male gametophyte development and the reduced fertility observed is most likely due to the effect of salt on stamen function.

To determine if altered pistil development or function also contributes to sterility in the *cbl10* mutant, wild-type and *cbl10* flowers were emasculated at stage 12 (Smyth et al., 1990), and pistils from control and salt-treated plants were pollinated with wild-type pollen from plants grown in control conditions. No seed developed in pistils from salt-treated *cbl10* plants, indicating that pistil function is also affected (Fig. 4A). To determine which processes (e.g. pollen germination, tube growth in the transmitting tract, tube growth toward the ovules [targeting], or fusion of the gametes) were affected during fertilization, wild-type and *cbl10* pistils were pollinated with wild-type pollen expressing *GUS* under the control of the pollen-specific *LAT52* promoter (Johnson et al., 2004). Several defects were observed in pistils from salt-treated *cbl10* plants; in some pistils, pollen was unable to germinate or grow (Fig. 4B), while in others pollen tube growth was inhibited upon entering the transmitting tract of the ovary or shortly thereafter (Fig. 4B). Because the *cbl10* flower phenotypes are not evident at stage 12 (Smyth et al., 1990), it was not possible to link the differences in pistil defects to the distinct *cbl10* phenotypes. To determine if transmitting tract development in the *cbl10* mutant is impaired, sections of pistils from stage 14 flowers (Smyth et al., 1990) were stained with Alcian Blue to detect polysaccharides present in the transmitting tract of the mature pistil. No difference in Alcian Blue staining was detected in wild type or the *cbl10* mutant, indicating that the defect in pistil development is independent of or occurs after the secretion of polysaccharides into the extracellular matrix of the transmitting tract (Fig. 4C). One difference observed was the apparently increased diameter of pistils from salt-treated *cbl10* plants relative to wild-type plants or *cbl10* plants grown in control conditions (Fig. 4C).

Pollen tubes that were able to enter the transmitting tract of *cbl10* salt-treated plants did not show any signs of targeting the ovules (Fig. 4B). To determine if this is caused by a disruption in female gametophyte development, heterozygous *CBL10*^{−/−} mutant plants were allowed to self-pollinate, or emasculated flowers from these plants were pollinated with wild-type pollen. Seed from self-pollinated plants (untreated and salt-treated) and crosses (pollen from untreated plants used to pollinate pistils from salt-treated plants) was collected and seedlings genotyped to determine segregation of the mutant allele. The observed ratio of wild-type to mutant allele was not significantly different from the expected ratio (Supplemental Table S1), indicating that *CBL10* does not have a role in female gametophyte development and that the reduced fertility observed is most likely due to the effect of salt on pistil function.

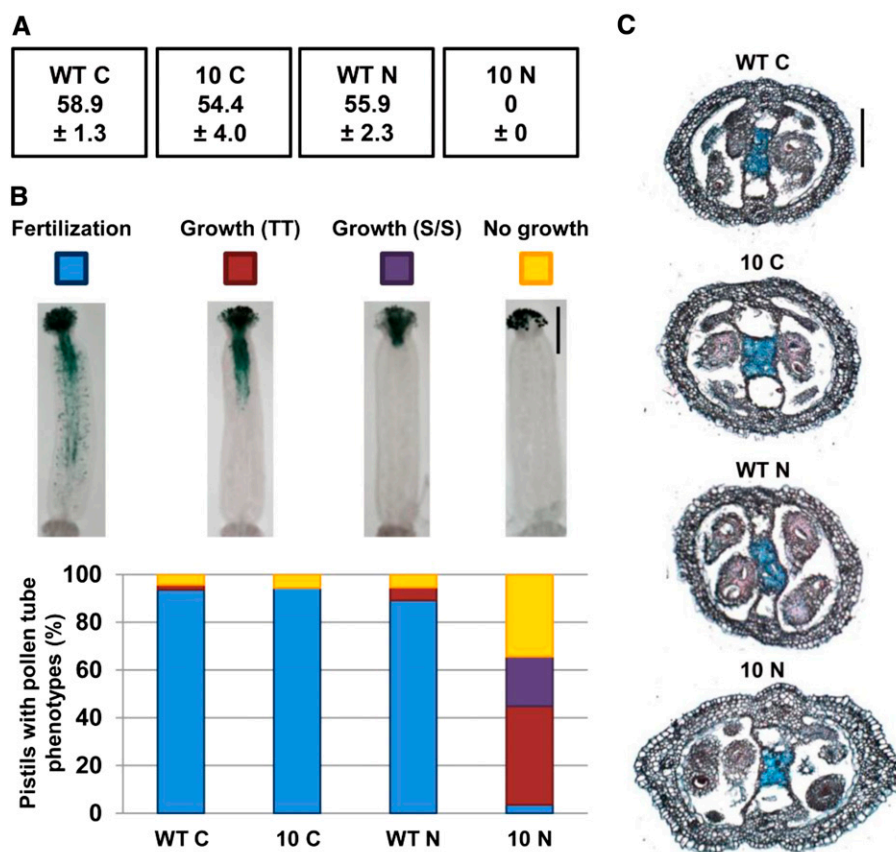


Figure 4. Pollen tube growth is impaired in *cbl10* pistils from salt-treated plants. **A**, Wild-type (WT) and *cbl10* (10) pistils from plants left untreated (control, C) or treated with salt (40 mM NaCl, N) were pollinated with wild-type pollen from plants grown in the absence of salt, and seed development was evaluated 10 d after pollination. The mean number of seed and the se are shown for 20 siliques per genotype per treatment from three independent experiments. **B**, Pistils were pollinated with wild-type pollen expressing *GUS* from a pollen-specific (*LAT52*) promoter, and pollen tube growth was examined. Top, Representative photographs of the pollen tube growth phenotypes. Bar (1 mm, right) shows magnification for all images. Bottom, Number of pistils with pollen tube growth phenotypes was graphed as a percent of the total number of pistils examined. Number of pistils analyzed: WT C, 52 pistils; 10 C, 58 pistils; WT N, 59 pistils; and 10 N, 94 pistils. Pistils were analyzed from plants grown in three independent experiments. TT, Transmitting tract; S/S, stigma and style. **C**, Wild-type and *cbl10* pistils were sectioned and treated with Alcian blue, which stains polysaccharides present in the transmitting tract. Number of pistils analyzed: WT C, six pistils; 10 C, five pistils; WT N, nine pistils; and 10 N, 14 pistils. Pistils were analyzed from plants grown in two independent experiments. Bar (50 μ m, top) shows magnification for all images.

Ion Levels Are Altered in the *cbl10* Mutant in Response to Salt

In leaves, CBL10 interacts with SOS2 to regulate sodium ion homeostasis by activating the SOS1 plasma membrane sodium/proton exchanger (Lin et al., 2009; Quan et al., 2007) and/or a vacuolar transporter (Kim et al., 2007). To determine if CBL10 functions to regulate sodium ion homeostasis during reproductive development, wild-type and *cbl10* plants were treated with isomolar concentrations of NaCl, KCl, NaNO₃, or KNO₃. Sterility in the *cbl10* mutant was observed in response to sodium but not potassium, chloride, or nitrate (Fig. 5), indicating that the phenotype is sodium-specific and the result of ionic stress.

Growth in saline conditions can result in a large increase in cellular sodium ion accumulation and a

decrease in calcium and potassium ion accumulation (Hasegawa et al., 2000). Calcium and potassium are known to be important for pistil and stamen development, and a disruption in the homeostasis of either ion can alter reproductive development (Ge et al., 2007; Heslop-Harrison et al., 1987; Mouline et al., 2002; Rehman and Yun, 2006). To determine if CBL10 regulates sodium levels to ensure that calcium and/or potassium ion homeostasis is maintained, the levels of sodium, calcium, and potassium were measured in wild-type and *cbl10* mutant flowers, pistils, and stamens using an inductively coupled plasma-mass spectrometer. In the salt-treated *cbl10* mutant, sodium levels increased and calcium levels decreased in all three tissues examined (Fig. 6). Even though potassium levels did not change, the ratio of cellular potassium to sodium decreased. The increase

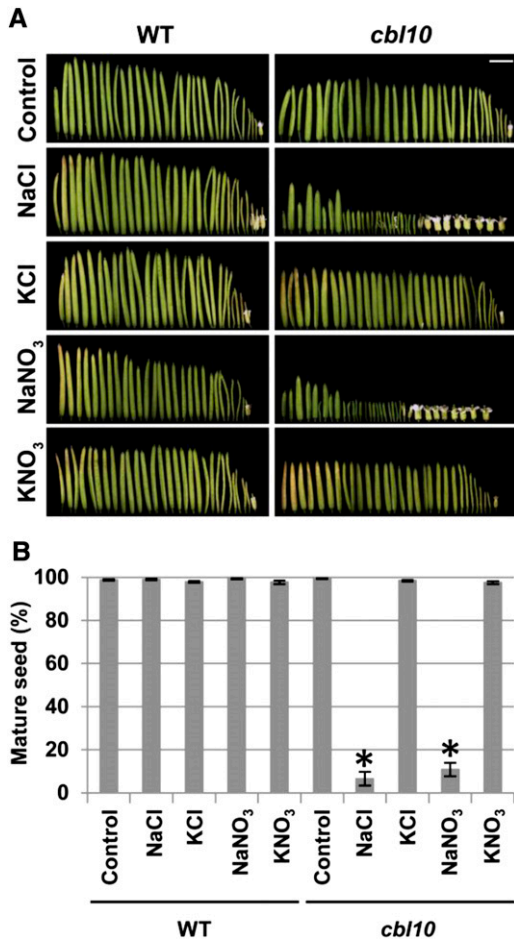


Figure 5. The *cbl10* sterile phenotype is due to sensitivity to sodium. A, Siliques from a primary inflorescence of representative wild-type (WT) and *cbl10* plants left untreated (Control) or treated with salt (40 mM of the indicated salt). Bar (5 mm, top right) shows magnification for all images. B, Seed development was analyzed in one 10-d-old silique per plant that developed on the primary inflorescence after 2 weeks of treatment. Twenty siliques were analyzed for each genotype per treatment from plants grown in three independent experiments. The number of seeds was graphed as a percent of the total number of positions within the silique. The data were rank transformed and an ANOVA conducted using a mixed model. Asterisk indicates that the number of *cbl10* seeds was significantly different ($P \leq 0.05$) than the number of wild-type seeds from plants treated with the same salt. Bars represent se.

in sodium was specific to floral tissues (Supplemental Fig. S5).

When salt-sensitive plants are grown in the presence of salt, addition of potassium and calcium has been shown to restore plant growth (Amtmann et al., 2001; Benlloch et al., 1994; Caines and Shennan, 1999; Cramer et al., 1985; Lahaye and Epstein, 1969; Morgan et al., 2014; Rubio et al., 2009; Shabala et al., 2005; Turhan et al., 2013; Zhong and Lauchli, 1994). To determine if addition of potassium and calcium can ameliorate the *cbl10* sterile phenotype, KCl and CaCl₂ were added with the NaCl treatments. Both were able to reduce the severity of the reproductive defects in the *cbl10* mutant

as indicated by an increase in the length of the siliques and the amount of seed produced in the mutant (Fig. 7). The addition of potassium had an effect throughout silique development, whereas calcium delayed the onset of sterility (Fig. 7A).

DISCUSSION

CBL10 Functions in Reproductive Development when Plants Are Grown in Saline Conditions

When *cbl10* plants were treated with 40 mM NaCl, 90% of the ovules failed to develop into seeds (Fig. 1), indicating that CBL10 functions early in reproductive development before fertilization. Analysis of floral organ development and function indicated that CBL10 is important for multiple processes when plants are grown in saline conditions. In the salt-treated *cbl10* mutant, anthers did not dehisce (Fig. 3), stamens were short (Fig. 3), pollen development ceased (Fig. 3), and wild-type pollen tube growth was arrested in *cbl10* pistils (Fig. 4). Several lines of evidence indicate that CBL10's role in reproductive development in salt is not due to defects in vegetative growth under our treatment conditions. Loss of CBL10 activity had no effect on vegetative or reproductive development during growth in control conditions and only affected reproductive (seed) development when plants were treated with salt at the start of inflorescence development (Fig. 1). All other parameters measured in the *cbl10* mutant (rosette fresh weight, inflorescence height, number of siliques, and number of positions within a silique) were similar to what was found in wild type in both control and salt-treated conditions (Supplemental Fig. S1). In addition, rosette fresh weight was maintained through the end of the salt treatment in the *cbl10* mutant (Supplemental Fig. S1).

CBL10 Functions Independently of the SOS Pathway When Plants Are Grown in Saline Conditions

During vegetative development, CBL10 interacts with SOS2 to regulate sodium ion homeostasis either by activating the SOS1 plasma membrane sodium/proton exchanger (Lin et al., 2009; Quan et al., 2007) or a vacuolar transporter (Kim et al., 2007). Several results indicate that CBL10 functions during reproductive development independently of SOS2 and SOS1. First, loss of SOS2 activity had no effect on seed development, indicating that SOS2 does not have a role in reproductive development in response to salt (Fig. 1; Supplemental Fig. S1). Second, salt affected reproductive development differently in the *sos1* and *cbl10* mutants. In response to salt, the total number of seed positions within a *sos1* silique decreased (Fig. 1; Supplemental Fig. S1), and the number of aborted seed and unfertilized ovules increased. In contrast to *cbl10* in which 90% of the ovules were unfertilized in response to salt, only 30% of the *sos1* ovules were unfertilized. The majority of the *sos1* ovules either matured (46%) or

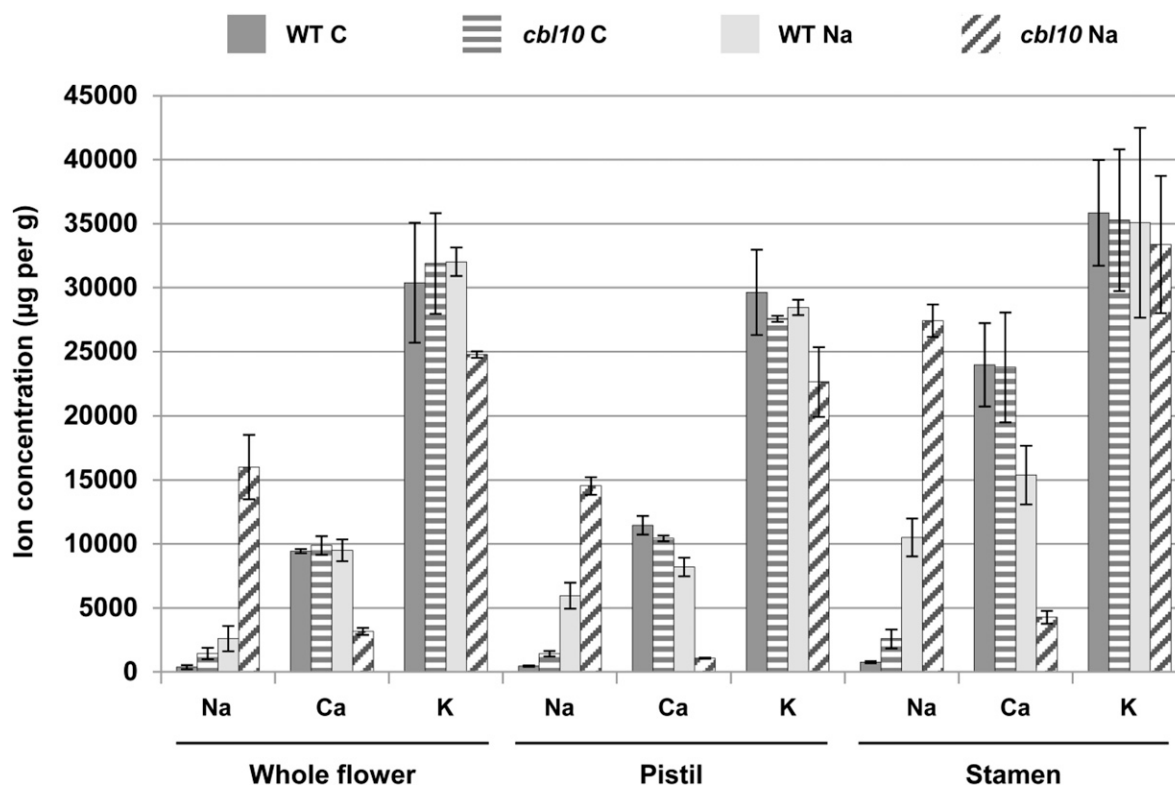


Figure 6. Ion homeostasis is altered in flowers, pistils, and stamens of salt-treated *cbl10* plants. Flowers (stage 14; Smyth et al., 1990), pistils, and stamens were harvested from wild-type (WT) and *cbl10* left untreated (control, C) or treated with salt (40 mM NaCl, Na). The mean concentration of ions ($\mu\text{g per g}$ of tissue) from two independent experiments is shown. Bars represent se.

aborted early (24%), indicating that SOS1 likely functions largely after fertilization when seed development begins (Fig. 1).

CBL10 Likely Functions in Ion Homeostasis in Response to Ionic Stress

Salt affects plant growth and development in several ways; the accumulation of salt in the soil reduces water uptake into the plant (osmotic stress), while the accumulation of sodium within plant cells interferes with metabolic processes (ionic stress; Munns and Tester, 2008). While osmotic stress can cause sterility in *Arabidopsis* (Su et al., 2013; Sun et al., 2004), several lines of evidence suggest that the *cbl10* sterile phenotype is due to ionic stress. (1) The phenotype is specific to sodium; other salts, including KCl, did not cause sterility (Fig. 5). (2) Additional salt in the form of KCl and CaCl_2 ameliorated the phenotype rather than enhancing it (Fig. 7). (3) Ion homeostasis is disrupted in the *cbl10* mutant (Fig. 6). (4) CBL10 regulates sodium ion homeostasis in vegetative tissues (Kim et al., 2007; Lin et al., 2009; Quan et al., 2007). Additional evidence for CBL10's likely role in the regulation of ion homeostasis during reproductive development is based on the phenotypic similarities between the *cbl10* mutant and mutants in the SODIUM/HYDROGEN EXCHANGER1 (NHX1) and NHX2 potassium/proton exchangers and the

Cation-Chloride-Cotransporter (CCC) protein (likely involved in transporting potassium, sodium, and chloride) in *Arabidopsis*. Mutations in the NHX1 and NHX2 transporters led to reductions in filament elongation and anther dehiscence as well as to sterile pistils (Bassil et al., 2011). When the *Arabidopsis* CCC protein was mutated, flowers were sterile (Colmenero-Flores et al., 2007). The NHX and CCC genes appear to regulate ion homeostasis when plants are grown in control conditions, while CBL10 appears to prevent a disruption in ion homeostasis when plants are grown in the presence of salt (Fig. 6).

The *cbl10* mutant accumulates more sodium in its flowers compared with wild type when plants are treated with salt (Fig. 6). Two potential explanations for this pattern of accumulation include a function for CBL10: (1) in vegetative tissues sequestering sodium before it enters reproductive tissues and/or (2) in floral organs facilitating the movement of sodium out of reproductive tissues. Two observations support a direct role for CBL10 in floral organs; CBL10 is expressed in stamens and pistils (Fig. 2), and SOS2, which functions with CBL10 in leaves (Kim et al., 2007; Quan et al., 2007), does not have a sterile phenotype when mutated (Fig. 1).

Because increases in sodium levels in the plant are often correlated with decreases in calcium and potassium (Hasegawa et al., 2000) and these ions have been shown to be critical for reproductive development, changes in the levels or ratios of calcium and potassium

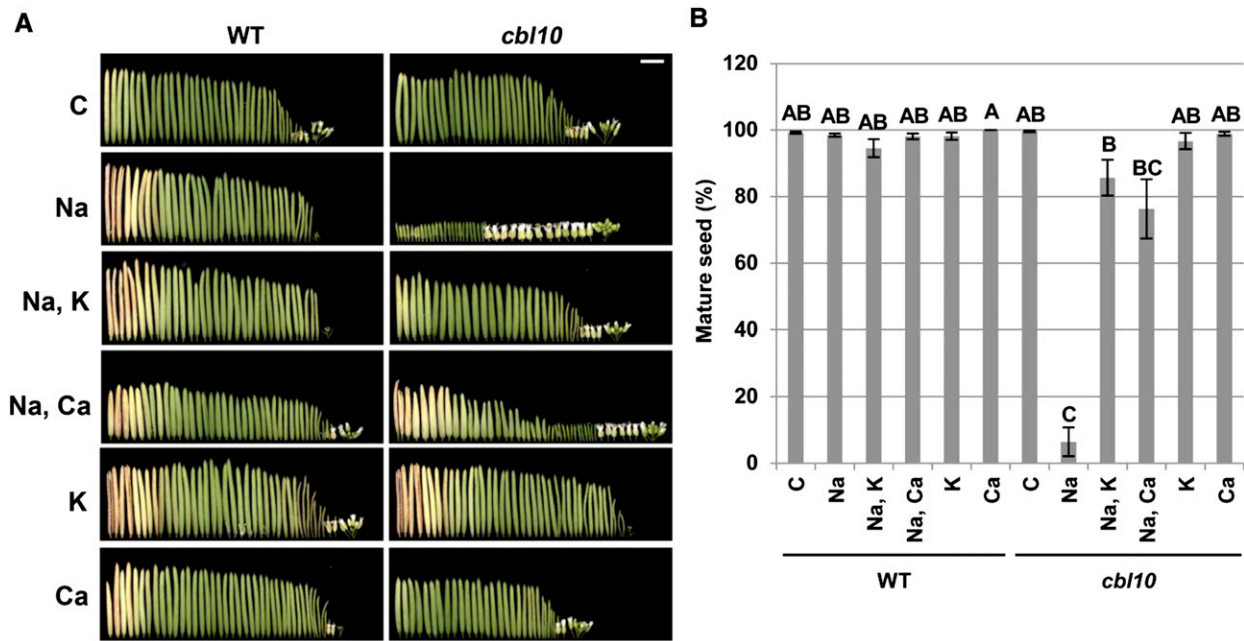


Figure 7. Potassium and calcium added during salt treatments ameliorate the *cbl10* sterile phenotype. For the amelioration experiments, potassium or calcium were added to wild-type (WT) and *cbl10* plants left untreated (control, C) or treated with salt (40 mM NaCl, Na). Potassium (40 mM) was added in the form of KCl to the control (K) and salt (Na, K) treatments, and calcium (20 mM) was added in the form of CaCl₂ to the control (Ca) and salt (Na, Ca) treatments. A, Siliques from a primary inflorescence (oldest to youngest, left to right). Bar (5 mm, top right) shows magnification for all images. B, One 10-d-old silique per plant that developed after 2 weeks of treatment was opened to monitor seed set. The number of mature seed per silique was graphed as a percent of the total number of positions in a silique. Siliques were examined in two independent experiments (mean: 13 siliques; range: 11–16). The data were rank transformed and an ANOVA conducted using a mixed model. Any pair of genotypes/treatments that do not share the same letter are significantly different ($P \leq 0.05$) based on a Tukey HSD significance test. Bars represent \pm SE.

may underlie the reproductive defects seen in *cbl10*. Calcium and potassium have been shown to be involved in: (1) cell elongation (Fuchs et al., 2006; Hepler, 2005; Heslop-Harrison et al., 1987), (2) anther dehiscence (Matsui et al., 2000; Rehman and Yun, 2006; Tian et al., 1998), (3) pollen grain development (Ge et al., 2007; Scott et al., 2004), and (4) pollen tube growth (Dresselhaus and Franklin-Tong, 2013; Fan et al., 2001; Zhao et al., 2004). A decrease in calcium levels and/or the ratio of potassium to sodium in flowers could result in the reduced anther dehiscence, decreased filament elongation, and aborted pollen grain development that were observed in *cbl10* flowers (Fig. 3). In addition, a change in the effective concentrations of these ions in *cbl10* pistils might prevent pollen grains from germinating or pollen tubes from growing in the transmitting tract (Fig. 4). Altered effective concentrations of these ions might also underlie the inability of *cbl10* flowers from salt-treated plants to close in the afternoon (Supplemental Fig. S3). While little is known about the mechanism underlying flower opening and closing, the importance of potassium and calcium has been shown in other reversible movements, including opening and closing of stomata (Kim et al., 2010) and of leaves from *Mimosa pudica* (Moran, 2007).

The addition of supplemental calcium or potassium to salt-affected soils can ameliorate the toxic effects of salt on plant growth. Wheat (Ca²⁺, Amtmann et al.,

2001), tomato (Ca²⁺, Caines and Shennan, 1999), cotton (Ca²⁺, Cramer et al., 1985; Zhong and Lauchli, 1994), common bean (*Phaseolus vulgaris*; Ca²⁺, Lahaye and Epstein, 1969; K⁺, Benlloch et al., 1994), broad bean (*Vicia faba*; Ca²⁺ and K⁺, Morgan et al., 2014), pepper (Ca²⁺ and K⁺, Rubio et al., 2009), barley (Ca²⁺, Shabala et al., 2005), and spinach (Ca²⁺ and K⁺, Turhan et al., 2013) are among the plants with improved growth with addition of calcium or potassium during salt treatments. In these studies, the addition of supplemental calcium reduced sodium levels and increased potassium and calcium levels. Supplemental calcium most likely ameliorates salt sensitivity by mitigating the toxic effects of sodium ions rather than any associated osmotic effects (Rengel, 1992). Several mechanisms have been proposed to explain how calcium might do this including: (1) stabilization of cellular membranes to prevent ion loss (Cramer et al., 1985; Lynch and Läuchli, 1988), (2) a reduction of sodium uptake through nonselective cation channels (Demidchik and Tester, 2002), and (3) an increase in the potassium/sodium ratio (Cramer et al., 1987; Liu and Zhu, 1997; Zhong and Lauchli, 1994). Much less is known about the ability of supplemental potassium to ameliorate the toxic effects of salt on plant growth and development. Supplemental potassium also reduces sodium levels and increases potassium and calcium levels, but the mechanisms underlying these changes are unknown.

While the molecular mechanism underlying the function of CBL10 in reproductive development is currently unknown, it likely involves different interaction partners. SOS2 belongs to the 25-member CBL-Interacting Protein Kinase (CIPK) family (Luan, 2009). CIPKs have been shown to interact with multiple CBL calcium sensors and the formation of different CBL-CIPK complexes is thought to contribute to specificity during calcium signaling (Luan, 2009). CBL10 might interact with and activate SOS2 in leaves to regulate sodium levels during vegetative development, but interact with and activate another CIPK in flowers to regulate sodium levels during reproductive development. Alternatively, CBL10 may interact with a protein outside of the CIPK family (Nozawa et al., 2001; Oh et al., 2008). Future studies will focus on identifying potential CBL10-interacting proteins that might function with CBL10 during reproductive development.

MATERIALS AND METHODS

Plant Material

Arabidopsis (*Arabidopsis thaliana*) Col-0 was used as wild type for this study. A CBL10 T-DNA insertion line (SALK_056042) was obtained from the Arabidopsis Biological Resource Center and backcrossed to wild type three times to remove insertions in other genes. Genomic DNA from the mutant was used as a template to identify wild-type and mutant alleles. A homozygous *cb110* mutant was identified in the first self-pollinated generation of backcross 3. To test for the presence of a T-DNA insertion, the LBA1 (5'-TGGTTCACGTAGTGGGC-CATC-3') and 056042_L (5'-TCTGCTATTCTTGGAACTCGA-3') primers were used. To identify alleles without a T-DNA insertion (wild-type alleles), the 056042_L primer was used with 056042_R (5'-CTGCCATAGACGCAAGATGA-3'). The *sos1-1* (Wu et al., 1996), *sos2-2* (Zhu et al., 1998), and *sos3-1* (Liu and Zhu, 1997) ethylmethane sulfonate mutants were provided by Dr. Jian-Kang Zhu. To verify that the sterility observed in *cb110* is due to a mutation in the CBL10 gene, the CBL10 cDNA (protein-coding sequence) was cloned into pEZT-NL (Drs. Sean Cutler and David W. Ehrhardt, Carnegie Institution of Washington, Stanford, CA) using the 5CBL10XhoI (5'-GCGCTCGA-GATGGAACAAGTTTCTCTAGAT-3') and 3CBL10B (5'-GGCGGATCCT-CAGTCTCAACCTCAGTGTG-3') primers, and expression was driven by the cauliflower mosaic virus 35S promoter. *cb110* plants were transformed using *Agrobacterium tumefaciens* strain LBA4404 via the floral dip method (Clough and Bent, 1998). Transformed seed was selected on 10 µg/mL glufosinate ammonium, and T2 lines with 75% resistance (single insertion) were chosen. Homozygous seed was obtained by screening T3 seed on glufosinate ammonium (Santa Cruz Biotechnology) to identify lines with 100% resistance.

Plant Growth

Seeds were sown on Sunshine Soil Mix #1 (SunGrow Horticulture), stratified for 2 d at 4°C in the dark, and transferred to a growth chamber at 21°C using a 16 h light/8 h dark (800 lm/ft²) photoperiod (long-day conditions). Plants were watered every 2 to 3 d with 0.5× Hoagland's solution (Hoagland and Arnon, 1938) with cobalt chloride in place of cobalt nitrate and at a final pH of 5.7 (adjusted with KOH). After 3 weeks of growth, at the start of inflorescence development, salt in the form of NaCl, KCl, CaCl₂, NaNO₃, and/or KNO₃ was added to the 0.5× Hoagland's solution. Plants were treated for an additional 2 to 3 weeks.

To analyze silique development, one representative flower per plant, formed at 2 weeks of treatment, was marked, allowed to develop for 10 d, and silique length was measured. Seed development was analyzed by classifying the ovule or seed within each silique as mature seed (containing a green, mature embryo), defective seed (containing a white or brown, aborted embryo), or an unfertilized ovule (ovule that was not fertilized or seed that aborted just after fertilization).

Statistical Analyses

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 throughout. Data were rank transformed using Microsoft Excel (function: RANK) followed by an ANOVA and Tukey's honestly significant difference (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 $P \leq 0.05$ throughout, and all tests of significance were two sided.

Analysis of CBL10 Expression during Flower Development

Flowers from 5-week-old wild-type plants grown under long-day conditions were staged according to Smyth et al. (Smyth et al., 1990) and collected in the following pools: (1) meristem formation and early flower development (stages 1–11), (2) anthesis (stages 12 and 13), and (3) fertilization (stages 14 and 15). RNA was isolated using the Qiagen RNeasy Plant Mini Kit (Qiagen Sciences) and treated with TURBO DNase (Invitrogen). After purification (RNeasy MinElute Cleanup Kit; Qiagen), RNA was used for cDNA synthesis (SuperScript III Reverse Transcriptase; Invitrogen). To monitor the CBL10 transcript, the CBL10-RT-F1 (5'-GATCAAGCTCTCTCACTGTC-3') and CBL10-RT-R1 (5'-GCGCTATGACAATCTCACTC-3') primers were used. To monitor transcript levels of control genes, the following primers were used: *ELONGATION FACTOR1-ALPHA*, loading control, EF1α(471F) (5'-TGAGCAGCTCTTC-TTGCTT-3') and EF1α(826R) (5'-CCACTGGCACCGTTCCAAT-3'); *ABORTED MICROSPORES* (AMS), early flower development marker, AMS_F (5'-TCGCTTGTCCAGGATAACC-3') and AMS_R (5'-TTCCAGCAACGAGT-TCTTACG-3'); and MYB21, late flower development marker, MYB21_F (5'-TAAACGAACCGGAAAAGTT-3') and MYB21_R (5'-GCGGCCGAA-TAGTTACCATAG-3').

Histochemical Analyses

CBL10 Promoter Activity

The 5' upstream region of CBL10 was transcriptionally fused to *GUS* as a reporter gene in pCAMBIA1381 (GenBank accession no. AF234302). A 2,214-bp fragment representing the CBL10 promoter was amplified using Col-0 genomic DNA as a template, Phusion High Fidelity DNA Polymerase (Thermo Scientific), and the PM5-AtCBL10-3PstI (5'-GCGCTGCAGCG-GCCAGCGACGATAAAATGGTT-3') and PM3-AtCBL10-5XhoI (5'-GCGCTCGAGGATTCATTCAAAAATCACAATCACAG-3') primers. The promoter-GUS construct was introduced into *Agrobacterium tumefaciens* strain GV3101 and subsequently into wild-type Arabidopsis via the floral dip method (Clough and Bent, 1998). Transformed seed was selected on 25 µg/mL hygromycin, and T2 lines with 75% resistance (single insertion) were chosen. Homozygous seed was obtained by screening T3 seed on hygromycin to identify lines with 100% resistance. Flowers along a single inflorescence were detached, petals and sepals were removed, and a small incision was made in each pistil to facilitate stain entry into the ovary. Pistils and stamens were immersed in 90% (v/v) acetone for 20 min, briefly rinsed in GUS buffer (Jefferson et al., 1987; 500 mM NaPO₄ buffer, pH 7, 50 mM ferrocyanide, 50 mM ferricyanide), and then placed in GUS staining solution (GUS buffer containing 20 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-GlcA in dimethyl sulfoxide; X-Gluc; Gold Biotechnology). Flowers were vacuum infiltrated for 15 min and then incubated in staining solution overnight at 37°C. Flowers were removed from stain and cleared in 70% (v/v) ethanol for 5 h before mounting on slides with 50% (v/v) glycerol. Bright-field images were captured using an Olympus SZX12 stereomicroscope.

Alexander Staining of Pollen

Anthers were stained with Alexander's stain according to Peterson et al. (Peterson et al., 2010). Briefly, tissue was fixed in Carnoy's fixative (6 parts ethanol:3 parts chloroform:1 part acetic acid) for 2 d, dried on a paper towel,

immersed in Alexander's stain (5 mL of 95% (v/v) ethanol, 0.5 mL of 1% (w/v) Malachite Green oxalate [M9015; Sigma-Aldrich], 12.5 mL of glycerol, 2.5 mL of 1% (w/v) acid fuchsin [F8129; Sigma-Aldrich], 0.25 mL of 1% (w/v) Orange G [O7252; Sigma-Aldrich], 2 mL of glacial acetic acid with sterile distilled water added for a total volume of 50 mL), heated in an 80°C water bath for 1 min, and then incubated at room temperature for 2 min. Tissue was mounted on slides with 50% (v/v) glycerol and bright-field images captured using a Zeiss Axiophot microscope.

Pollen Tube Growth through Pistils

Preanthesis flowers (stage 12; Smyth et al., 1990) were emasculated, and pistils were allowed to mature for 20 h. Pistils were pollinated by brushing anthers from wild-type flowers expressing *GUS* under the control of the pollen-specific *LAT52* promoter (*LAT52pro:GUS*) against the pistil to release pollen onto the stigma. After 18 h, pistils were detached and submerged in 80% (v/v) acetone for 30 min. The pistils were then dipped in *GUS* buffer before being placed in *GUS* staining solution followed by incubation at 37°C overnight. Pistils were then submerged in fixing solution (6 parts 100% ethanol:1 part acetic acid) overnight at room temperature, followed by 100% ethanol for 30 min and then 70% (v/v) ethanol for 30 min. The ethanol was replaced with a clearing solution (4 parts chloral hydrate [g]:1 part 100% glycerol [mL]:2 parts sterile distilled water [mL]) overnight at room temperature. Pistils were mounted in the clearing solution on slides. Bright-field images were captured using an Olympus SZX12 stereomicroscope.

Alcian Blue Staining of the Transmitting Tract

Stage 14 flowers (Smyth et al., 1990) were fixed in a phosphate-buffered glutaraldehyde solution (25 mM phosphate buffer, pH 6.8, 2% (v/v) glutaraldehyde, 0.1% (v/v) Triton X-100), vacuum infiltrated five times for 15 min in a desiccator, and incubated at room temperature for 3 h and then at 4°C overnight. Fixed flowers were washed five times in 25 mM phosphate buffer, pH 6.8, dehydrated through an ethanol series (5%, 20%, 35%, 50%, 65%, 80%, and 95% (v/v) for 30 min each at room temperature), and incubated in 95% (v/v) ethanol containing 0.1% (w/v) Eosin Y (E4009; Sigma-Aldrich) at room temperature overnight. Samples were washed two times with 100% ethanol and cleared with a xylene:ethanol series (25% (v/v) xylene and 75% ethanol, 50% xylene and 50% ethanol, 75% xylene and 25% ethanol, and 100% xylene for 30 min each at room temperature). Tissue was embedded in Paraplast (23-021-400; Fisher Scientific), sectioned at 5 microns, and mounted on precoated slides (12-550-15; Fisher Scientific). Slides were dewaxed with 100% xylene, rehydrated through an ethanol series (100%, 75%, 50%, and 25% for 5 min each), briefly rinsed in sterile distilled water and then 3% (v/v) acetic acid, stained in 1% (w/v) Alcian Blue 8GX (05500; Sigma) and 3% (v/v) acetic acid for 2 h, and counterstained in 1% (w/v) neutral red (72210; Sigma-Aldrich) for 15 s. Slides were dried at room temperature and bright-field images captured using a Zeiss Axiophot microscope.

Ion Analysis

Whole flowers, pistils, and stamens were collected and dried for 2 d at 60°C. Samples were sent to the Arizona Laboratory for Emerging Contaminants at the University of Arizona for analysis using an inductively coupled plasma-mass spectrometer.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AF513507.2.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Inflorescence development and vegetative growth in the *cb110* mutant is not affected by salt treatments.

Supplemental Figure S2. *CBL10* complements the *cb110* sterile phenotype.

Supplemental Figure S3. Flowers from *cb110* mutant plants treated with salt do not close.

Supplemental Figure S4. Flowers on a single inflorescence of salt-treated *cb110* plants have multiple sterile phenotypes.

Supplemental Figure S5. Sodium accumulation was higher in flowers than in leaves in the salt-treated *cb110* mutant.

Supplemental Table S1. Transmission of the *cb110* allele through the male and female gametophyte is unaffected.

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