DR SORAYA PELAZ (Orcid ID : 0000-0001-7699-9330)

Article type : Original Article

The floral repressors TEMPRANILLO1 and 2 modulate salt tolerance by regulating hormonal components and photo-protection in *Arabidopsis*

Michela Osnato[1], Unai Cereijo[1], Jan Sala[1], Luis Matías-Hernández[1], Andrea E. Aguilar-Jaramillo[1], María Rosa Rodríguez-Goberna[1], José Luis Riechmann[1,2], Manuel Rodríguez-Concepción[1] and Soraya Pelaz[1,2,*].

[1]Centre for Research in Agricultural Genomics (CRAG), CSIC-IRTA-UAB-UB, Campus UAB, 08193, Barcelona, Spain.
[2] Institució Catalana de Recerca i Estudis Avançats (ICREA), 08010, Barcelona, Spain
*Author for contact: soraya.pelaz@cragenomica.es

Running title: TEM-salinity tolerance

Keywords: Abiotic stress, *Arabidopsis thaliana*, Flowering time, Plant development, Plant physiology, RAV genes, Salt tolerance, TEMPRANILLO

SUMMARY

Members of the plant specific RAV family of transcription factors regulate several developmental and physiological processes. In the model plant *Arabidopsis thaliana*, the RAV TEMPRANILLO 1 (TEM1) and TEM2 control important phase changes such as the juvenile to adult and the vegetative to reproductive transitions. Besides their known regulatory function in plant development, a transcriptomics analysis of transgenic plants overexpressing *TEM1* also revealed over-representation of GO categories related to abiotic stress response. Therefore, to investigate

the biological relevance of these TEM-dependent transcriptomic changes and elucidate whether *TEM* contribute to the modulation of plant growth in response to salinity, we analysed the behavior of *TEM* gain and loss of function mutants subjected to mild and high salt stresses at different development stages. With respect to increasing salinity, *TEM* over-expressing plants were hypersensitive whereas the *tem1 tem2* double mutants were more tolerant. Precisely, *tem1 tem2* mutants germinated and flowered faster than the wild type plants under salt stress conditions. Also, *tem1 tem2* plants showed a delay in salt-induced leaf senescence, possibly as a consequence of down-regulation of Jasmonic Acid (JA) biosynthetic genes. Besides a shorter life cycle and delayed senescence, *tem1 tem2* mutants appeared to be better suited to withstand oxidative stress as they accumulated higher levels of α-tocopherol (an important antioxidant metabolite) and displayed a slower degradation of photosynthetic pigments. Taken together, our studies suggest novel and crucial roles for TEM in adaptive growth as they modulate plant development in response to environmental changes such as increasing soil salinity.

INTRODUCTION

Plants are exposed to different environmental changes over the course of their life and employ several mechanisms to perceive external fluctuations and modulate their internal developmental programs. Nowadays, plants are facing ever-changing ecosystems and should adapt their growth to variable conditions associated with climate change to survive. In addition, the combination of elevated ambient temperature and intensive farming is leading not only to decreased water availability but also to increased concentration of Sodium Chloride (NaCl) in the soil. Thus, water shortage and salinity constitute the main threats to plant productivity, as both inhibit plant growth and could lead to plant death. Therefore, the elucidation of the molecular mechanisms underlying abiotic stress response is of great significance for the future of agriculture as novel findings could be used to develop engineered crops with enhanced resilience to climate change (Tester and Langridge, 2010).

General strategies for salinity tolerance include the regulation of both osmotic and ionic components of salt stress (Deinlein et al., 2014). These responses are triggered upon stress sensing likely mediated by calcium signaling, and specifically via increased levels of free Ca^{2+} in the cytosol (Tracy et al., 2008). When roots come into contact with high salt concentration in the soil, plant cells activate the production of osmolytes such as proline to maintain osmotic homeostasis

(Székely et al., 2008). Afterwards, plant cells manage to exclude toxic Na^+ ions from the cytoplasm to regulate ion homeostasis, both in the roots and in the shoot (reviewed in Deinlein et al., 2014).

In the model species *Arabidopsis thaliana*, the stress signal is transmitted into the cells via a peak of Ca^{2+} which activates the SALT OVERLY SENSITIVE (SOS) signaling pathway (Park et al., 2016). Upon perception of the Ca^{2+} spike, the ion-binding sensor SOS3 forms a complex with SOS2, a serine/threonine type protein kinase, which activates the plasma membrane Na^+/H^+ antiporter SOS1, for the exclusion of Na^+ from the cytosol (Apse et al., 1999; Liu et al., 2000; Shi et al., 2000; Qiu et al., 2002; Shi et al., 2002). Besides imbalance of osmotic and ionic homeostasis, salt stress also induces the accumulation of Reactive Oxygen Species (ROS) such as hydrogen peroxide and nitric oxide, which cause severe oxidative damage in plant cells due to peroxidation and de-esterification of membrane lipids (Baier et al., 2005). For this reason, additional cellular mechanisms underlying abiotic stress response include the activation of ROS detoxifying enzymes (e.g. superoxide dismutase, ascorbate peroxidase, catalases) and the production of ROS scavenging metabolites (e.g. ascorbate, glutathione, tocopherols) which provide protection to the cell (Neill et al., 2002). Eventually, stressed plants undergo premature senescence as a consequence of the activation of enzymes which catalyse the breakdown of chlorophylls, leading to impaired photosynthesis and leaf yellowing (Matile et al., 1996; Lim et al., 2003; Lim et al., 2007; Woo et al., 2019). Ultimately, most of the plants continuously exposed to high salinity die before setting seeds as salt shock leads to plasmolysis.

Despite the extensive knowledge in physiological strategies underlying salt stress response, less is known regarding the molecular mechanisms regulating growth adaptation to salinity. So far, only few genes have been described as regulators of plant development under unfavorable conditions in *Arabidopsis* (reviewed by Golldack et al., 2014). Among these, a group of TFs belonging to the GRAS family called DELLAs play pivotal roles in the negative regulation of growth in response to salinity by restraining cell proliferation and expansion (Achard et al., 2006; Achard et al., 2008). Indeed, salt stressed plants display reduced levels of the phytohormone gibberellic acid (GA), and consequently increased accumulation of DELLAs, which results in the reduction of leaf production, leaf expansion and biomass accumulation (Achard et al., 2006). Also, DELLAs mediate salt-induced delay of the floral transition by repressing the floral meristem identity gene *LEAFY* (Achard et al., 2006).

Additional negative regulators of general growth belong to the RAV sub-family of TFs. Indeed, transgenic plants over-expressing *RAV1*, *RAV2* (also known as *TEMPRANILLO2; TEM2)* and the related *RAV2-like* gene (also known as *TEMPRANILLO1; TEM1*) display growth defects such as dwarfism and loss of apical dominance (Castillejo and Pelaz, 2008; Fu et al., 2014). *TEM* over-expressors (*TEM-OE*) exhibit specific developmental alterations, such as extremely late flowering under inductive long days (LD) and glabrous leaves, due to very reduced transcription of different direct targets including the florigens *FLOWERING LOCUS T (FT)* and *TWIN SISTER OF FT (TSF1),* and genes encoding trichome initiation factors (Castillejo and Pelaz, 2008; Osnato et al., 2012; Marín-González et al., 2015; Matías-Hernández et al., 2016). Conversely, loss of function mutants show developmental alterations related to increased content of GA as for instance longer hypocotyls, early flowering under non inductive short days (SD) and higher number of trichomes (Osnato et al., 2012; Matías-Hernández et al., 2016). Unlike the *DELLA* loss of function mutants which are more sensitive to high salinity, mutants characterised by T-DNA insertions in *RAV1*, *RAV1-like* and *RAV2*/*TEM2*, are more tolerant to salt stress during the vegetative phase (Fu et al., 2014).

Here, transcriptomics analysis of *TEM1-OE* transgenic plants revealed enrichment in GO terms related to abiotic stress response. To further investigate the role of TEMs in the modulation of plant growth in response to salinity, we exposed gain and loss of function mutants to increasing salt concentration in order to simulate natural incidences of salinity in agricultural and/or natural ecosystems. Indeed, salt accumulation occurs gradually in the soil as water content decreases because of seasonal drying, evaporation and transpiration. Our studies indicated that *TEM1-OE* plants were hypersensitive to salinity whereas *tem1 tem2* double mutants were more tolerant than wild-types to mild and high salt stresses, likely due to alterations of hormonal pathways. Under salt stress, *tem1 tem2* mutants performed better than wild-type plants in fundamental developmental processes (such as germination, vegetative growth and flowering time), and salinity-induced senescence since they tolerated better the oxidative damage. Because *tem* mutants grow similarly in presence or absence of salt, TEMs seem to be required for the reduced vegetative growth and delayed floral transition under salt stress. Our findings suggest a role for TEMs as negative regulators of plant development in response to salinity.

RESULTS

Analysis of *TEM1-OE* transcriptome reveals mis-regulation of abiotic stress-related genes

To identify additional genetic determinants and pathways acting downstream of TEM1, we investigated the transcriptomics changes between *TEM1-OE* and wild-type plants using RNA-sequencing. Among almost 3000 differentially expressed genes (DEGs, Dataset I), the number of up-regulated genes was slightly higher compared with the number of down-regulated genes (1625 up-regulated genes representing 54%; 1372 down-regulated genes representing 46%).

A preliminary singular enrichment analysis (SEA) for biological processes revealed an over-representation of GO categories related to vegetative development in the group of genes down-regulated in *TEM1-OE* (Figure S1a), in agreement with the known role of TEM1 as negative regulator of plant growth. Additional analyses showed enrichment of GO terms correlated with the response to endogenous stimuli, in particular to the stress hormone JA among the down-regulated genes (Figure 1a) and confirmed the enrichment of terms associated with abiotic stress (Figure S1b, Figure 1b), specifically "water deprivation" in the group of up-regulated genes in *TEM1-OE* plants. Therefore, the comparison of wild-type and *TEM1-OE* transcriptomes indicated mis-regulation of multiple genes and pathways related to stress response in transgenic plants, suggesting that TEM could have additional roles in the adaptation to adverse environmental conditions, likely modulating the biosynthesis of phytohormones. Previous studies demonstrated that GA_4 content was lower in *TEM1-OE* plants as a result of the direct repression of GA biosynthetic genes, and conversely higher in *tem1 tem2* mutants due to the de-repression of direct targets (Osnato et al., 2012; Matías-Hernández et al., 2016). Moreover, *tem1 tem2* mutants displayed lower levels of JA (Figure S2a). Interestingly, mapping of DEGs in *TEM1-OE* to the Kyoto encyclopedia of genes and genomes (KEGG) database revealed enrichment of hormone signal transduction pathways converging on the plant growth regulator GA, and also on the senescence-related hormone JA (Figure S2b). Eventually, KEGG pathway enrichment analysis uncovered components correlated with defense response and adaptation to abiotic stresses (Figure S2c). Therefore, these results opened the possibility that *TEM* genes could be involved in salt stress responses, for that reason we decided to thoroughly study the different developmental aspects affected by salinity in *TEM* gain and loss of function mutants.

Plants mis-expressing *TEM* genes display different sensitivity to salt stress

Previous work indicated that members of the RAV family of TFs in *Arabidopsis* could play a dual role in the control of plant development and in the response to abiotic stresses, and specifically in

tolerance to salt stress in an ABA-independent manner during the vegetative phase (Fu et al., 2014). To investigate the possible function of TEMs in the modulation of plant growth in response to salinity, we treated wild-type and transgenic plants mis-expressing *TEM* genes with water containing 50 mM and 100 mM of NaCl to simulate mild and high salt stress conditions, respectively (Shavrukov, 2013). Given the functional redundancy between *TEM1* and *TEM2,* we tested the *tem1 tem2* double mutants (hereafter, *tem*) along with *TEM1-OE* plants. In response to increasing soil salinity, we found higher mortality of *TEM1* gain of function mutants and conversely higher survival of *tem* loss of function mutants as compared to wild type plants (Figure 2a). Visual examination of four weeks-old plants confirmed that the diameter of the wild-type rosettes decreased in response to increasing salt concentration (Achard et al., 2006; Fig. 2c). However, *tem* plants, which had flowered early and their rosette leaves had ceased growth, seemed to be less sensitive to salt stress (Figure 2b), as their reduced leaf surface might have enabled them to overcome the negative effects of salinity on general growth. Nevertheless, *TEM1-OE* plants seemed to be hypersensitive to salt despite their small size, and displayed alterations of rosette leaves color, which suggested senescence-associated death of mesophyll cells (Figure 2c). Therefore, *TEM* genes may have a genuine role in salt sensitivity. Wild-type plants showed clear retardation of vegetative growth and flowering five weeks after salt treatment (Figure 2d), whereas most of the mutant plants achieved the reproductive phase when the stress intensified (Figure 2e). Therefore, these phenotypic analyses suggested that *tem* plants are less sensitive to increasing NaCl concentrations and show higher reproductive success than wild type plants.

TEMs mediate the floral transition in response to salt stress

To investigate the putative connection between salt stress and TEM floral repressors, we analysed flowering time of wild-type versus *tem* plants in response to increasing salinity. Under SD, wild-type plants watered with low and intermediate levels of NaCl failed to reach the reproductive phase, whereas the early flowering *tem* mutants underwent the floral transition under mild salt stress at the same time as in control conditions (Figure S3a). Under LD, wild-type plants subjected to mild salt stress flowered later than control plants, while half of the wild-type plants subjected to high salt stress died before flowering (Figure 3a-b, Figure S3b). By contrast, *tem* mutants showed only slightly inhibited growth and flowered within 3 weeks from germination with 6 leaves on average regardless of the treatment (Figure 3a-b, Figure S3b). Surprisingly, most of the mutants also produced flowers under salt shock under inductive day lengths (Figure S3c). Thus, the

decreased sensitivity of loss of function mutants to salt stress at floral transition suggested that *TEM* genes might mediate the delayed vegetative to reproductive phase change in response to salinity. Supporting this, wild-type plants treated with salt accumulated higher mRNA levels of *TEM1* and *TEM2* during the vegetative phase (Figure S4a). However, we did not detect alteration of transcription of the floral activator *CONSTANS (CO,* Putterill et al., 1995) in our experimental conditions (Figure S4b).

To further investigate the molecular basis of decreased salt sensitivity of *tem* mutants at floral transition, we examined changes in the transcription of genes acting downstream of TEM in plants grown under LD for one week. The expression of *FT* and *TSF* decreased as salt concentration increased in the two genotypes, but higher transcripts levels of the florigens (Fold Change=4) were found in *tem* mutant in all conditions (Figure 3c, Figure S4c). We also observed up-regulation of the GA biosynthetic gene *GA3-OXIDASE 1* (*GA3OX1*) and the floral integrator *SUPRESSOR OF CONSTANS OVEREXPRESSION 1* (*SOC1,* Samach et al., 2000) in the mutant background (Fold Change=2) in controlled and salt stress conditions (Figure 3d, Figure S4d). Therefore, phenotypic and expression analyses indicated a correlation between the early flowering phenotype and the de-repression of floral integrators and GA biosynthetic gene in *tem* plants irrespective of the treatment. In conclusion, a shorter life cycle appeared to be advantageous for mutant plants, which completed the reproductive phase even under severe salt stress (Figure S3c).

Early stages of vegetative growth under salt stress are less affected in *tem* mutants

Since germination is a physiological process affected by salinity, we followed the emergence of the radicle from imbibed seeds sown on medium containing low and intermediate levels of NaCl. As expected, the germination rate decreased as salt concentration increased for wild-type and *tem* seeds (Figure S5a). Both genotypes behaved similarly except three days after imbibition at 100 mM NaCl, when *tem* showed a significant higher percentage of germinated seeds (Figure S5a). This finding is in agreement with previous studies showing that seed germination in response to high salt stress is promoted in *rav* mutants and inhibited in plants over-expressing *RAV1*, *RAV1-like* and to a lesser extend *RAV2* (Fu et al., 2014). We reasoned that increased GA levels in the mutant (Osnato et al., 2012) could explain the promotion of *tem* seedling establishment. As supporting evidence, germination rates of seeds sown on the same media supplemented with 1 μM GA_3 were recovered to control MS rates (Figure S5b). At the molecular level, we observed increased expression of *ABA INSENSITIVE 5* (*ABI5*) and decreased expression of *GA3OX1*,

negative and positive regulators of seed germination respectively (Skubacz et al. 2016, Kim et al., 2008), in response to increasing salt concentration (Figure S5c-d). Nevertheless, *tem* seeds germinating on salt-containing medium showed higher transcript levels of *GA3OX1* than wild-type counterpart (Figure S5d). Therefore, higher GA content - endogenous in the mutant or exogenous in the medium - could compensate salt-induced inhibition of seed germination.

Next, we monitored the inhibitory effect of salinity on the growth of wild-type and mutant seedlings and observed visible reduction in root development and cotyledon expansion in both genotypes in response to salt treatment (Figure S6). Despite the variability among biological replicates, we also found statistically significant increase in primary root length in 1-week-old mutant seedlings (Figure S6b) but not in cotyledon diameter (Figure S6c). Similarly, we observed a reduction in rosette area of 2-week-old seedlings in response to increasing salinity but no statistical difference between genotypes (Figure S7a-b). Furthermore, we measured the fresh weight of seedlings subjected to salt treatment, when both genotypes are at the same developmental stage (i.e., before bolting). We confirmed the negative correlation between biomass accumulation and salt concentration but observed higher fresh weight of mutant plants exposed to mild salt stress compared to wild types in the same condition (Figure S7c). This might be due to increased size of mesophyll cells or increased number of trichomes in *tem* mutant leaves, as previously described (Matías-Hernández et al., 2016).

Taking into consideration the developmental differences between the two genotypes associated with alterations of flowering time, *tem* seedlings seemed slightly less sensitive than wild types to increasing salinity during the juvenile phase.

tem mutants delayed the progression of salinity-induced senescence

Metabolic analyses indicated that *tem* mutants have higher content of bioactive GA_4 (Osnato et al., 2012; Matías-Hernández et al., 2016), which could compensate growth delay induced by salinity, and lower levels of JA (Figure S2a), a lipidic phytohormone involved in stress response and multiple developmental processes including leaf senescence (He et al., 2002). Therefore, we wondered if altered hormonal content could allow *tem* loss of function mutants to better cope with abiotic stress.

To analyze the relationship between JA signaling pathway and salinity-induced senescence in wild-type versus mutant plants, we first examined the expression of genes encoding key enzymes of JA biosynthesis, especially members of the Lipoxigenase (LOX) and Allene Oxide Cyclase

(AOC) families which are strongly induced in senescent leaves (He et al., 2002; Li et al., 2014) and up-regulated in *TEM1-OE* (Dataset I). Although their expression increased in response to salinity in seedlings, we observed drastic downregulation in *tem* mutants in all treatments (Figure 4a-b, Figure S8a-b). Likewise, we found similar expression patterns of *LOX* genes after floral transition (Figure 4c, Figure S8c). Strikingly, we could not detect expression of *LOX4* in the mutant background in any condition tested. Given that TEMs function as repressors of transcription, we speculated that TEM could indirectly regulate JA biosynthetic genes by controlling the expression of factors involved in the JA signal transduction pathway (Figure S2b).

To expand the molecular analysis of plants undergoing salinity-induced senescence, we selected few candidate genes based on their mis-regulation in *TEM1-OE* (Dataset I), and their known function in signal transduction pathways activated in response to abiotic stresses (Noh and Amasino, 1999; Otegui et al., 2005; Xu et al., 2008; Zhou et al., 2009; Bhaskara et al., 2012; James et al., 2018; Nguyen and Cheong, 2018). We first monitored the transcription of *Mitogen-activated protein kinase kinase 9* (*MKK9)*, a positive regulator of leaf senescence (Zhou et al., 2009); *tem* plants displayed reduced *MKK9* expression under mild salt stress (Figure 4d, Figure S9a), suggesting that the progression of stress-induced senescence could be attenuated in the mutant.

An increased content of JA also induces the activation of several senescence associated genes (SAG; Gepstein et al., 2003; Liu et al., 2011). Among SAGs, we analyzed changes in the expression of genes encoding SAG113 (also known as Highly ABA-Induced1/HAI1*)*, a member of clade A Protein Phosphatases 2C modulated by salinity (Bhaskara et al., 2012; Nguyen and Cheong, 2018), and SAG12, a Cystein type peptidase with proteolytic activity involved in aging and leaf senescence (Noh and Amasino, 1999; Otegui et al., 2005; James et al., 2018). We found downregulation of *SAG113/HAI1* in *tem* mutants in all conditions (Figure 4e, Figure S9b), and strong activation of *SAG12* only in wild-type plants after 3 weeks of high salt stress (Figure 4f, Figure S9c).

To summarize, the molecular analyses of salt-stressed plants revealed down-regulation of selected JA biosynthetic genes and *SAGs* in the mutant, suggesting that senescence could be delayed in *tem* plants. Therefore, we hypothesized that TEMs could also act in the positive regulation of leaf senescence in addition to the negative regulation of plant growth.

tem mutants are better prepared to tolerate salt-induced oxidative stress

Abiotic stresses cause a plethora of alterations and promote the generation of free radicals - especially those derived from oxygen - which lead to serious damages of cellular membranes, impairment of ion fluxes and defective photosynthesis. Indeed, the excessive production of ROS contributes to the progression of leaf senescence, which can be activated by *MKK9* (Liu et al., 2008). As this gene was up-regulated in *TEM1-OE* plants, we wondered if leaf yellowing and the hypersensitivity observed in the gain of function mutant treated with salt could be associated to increased ROS accumulation. Therefore, we performed 3,3′-DiAminoBenzidine (DAB) staining to examine the presence of ROS in both gain and loss of function mutants (Figure 5a). Despite the strong detection of hydrogen peroxide (H_2O_2) in older leaves of all genotypes, salt-treated plants were stained more intensely by DAB than the control, supporting increased production of H_2O_2 in response to salinity. Moreover, the results from DAB staining were consistent with the hypothesis that *TEM1-OE* plants accumulated more ROS than the wild type (Figure 5a). On the contrary, *tem1 tem2* mutants showed less staining than wild types (Figure 5a), suggesting lower accumulation of ROS in response to salt stress, which correlated with the observed lower expression of genes involved in stress-induced senescence.

In order to withstand oxidative stress, plants have developed different molecular strategies to maintain the balance between ROS production and ROS scavenging, including the activation of enzymes involved in the detoxification and the biosynthesis of anti-oxidants (Neill et al., 2002). Interestingly, in our Dataset we found downregulation of *GAMMA-TOCOPHEROL METHYLTRANSFERASE* 1 (*G-TMT1*), also named *VITAMIN E DEFICIENT 4* (*VTE4*), a gene induced in response to abiotic stresses and encoding an enzyme involved in the metabolism of vitamin E (Bergmüller et al., 2003; Semchuk et al., 2009). Thus, the reduction in *G-TMT1/VTE4* expression in *TEM1-OE* plants could correlate with decreased content of antioxidants, increased content of ROS, and consequently accelerated leaf senescence in response to salt stress (Figure S9d). By contrast, *tem* mutants displayed higher expression levels of *G-TMT1/VTE4* (Figure 5b; Figure S9e), and accordingly higher accumulation of α-tocopherol in all conditions (Figure 5c). In addition, *tem* mutant exhibited increased expression of *EARLY LIGHT-INDUCIBLE PROTEIN 2* (*ELIP2*) (Figure 5d, Figure S9f), which encodes a protein able to bind free chlorophylls released during the assembly and turnover of light harvesting complexes (Hutin et al., 2003; Tzvetkova-Chevolleau et al., 2007). Taken together, these results suggested that *tem* mutants could be less sensitive to the salt-induced oxidative damage because of enhanced ROS detoxification and accumulation of molecules with photo-protective functions.

tem mutants display altered pigment degradation and NPQ kinetic in response to salinity

In addition to molecular markers associated to senescence and oxidative stress, the activation of well-established physiological indicators of chloroplast degeneration and consequent degradation of photosynthetic pigments marks the onset of senescence (Kuai et al., 2018). Therefore, we measured the content of chlorophylls and carotenoids in the two genotypes in different conditions to quantify the symptoms of impaired photosynthesis at maturity. Mutant plants displayed a lower content of chlorophylls and carotenoids under control conditions. However, the total content of these pigments declined rapidly in wild type but more slowly in *tem* mutants in response to salt stress (Figure 6a, Figure S9g, Table S1), suggesting that the progression of salinity-dependent leaf senescence was delayed in the mutant.

Since stressed plants undergo premature senescence to get rid of those leaves which are not photo-synthetically efficient, we decided to evaluate the photosynthetic performance of wild-type and mutant plants in response to salt stress by using a non-invasive phenotyping technology (PAM IMAGING, Table S2). Specifically, we calculated Non Photochemical Quenching (NPQ), a photo-protective mechanism developed to dissipate excess light energy from light harvesting complexes as heat (Ruban, 2016). Under controlled conditions, the two genotypes displayed similar behaviors: NPQ continued to increase during the exposure to actinic light and achieved their maximum after 9 minutes (Figure 6b). In response to increasing soil salinity, the two genotypes showed different NPQ kinetic profiles: wild-type plants reached higher values of maximum NPQ compared to *tem* mutants (Figure 6c, 6d). Furthermore, NPQ was unaltered in mutant plants subjected to mild and high salt concentrations, suggesting that *tem* plants could have a less efficient NPQ or alternatively experience reduced stress.

DISCUSSION

TEMs regulate adaptive growth in response to salt stress

Previous studies indicated that members of the RAV family control plant development and the response to water shortage and salinity in different plant species (Sohn et al., 2006; Li et al., 2015; Duan et al., 2016; Zhao et al., 2017), making them good candidates for the regulation of adaptive growth. Among *RAV* genes in *Arabidopsis*, *TEM1* and *TEM2* are widely expressed in different

tissues during the plant life cycle and play diverse roles in plant growth by negatively regulating the content of GA (Osnato et al., 2012; Matías-Hernandez et al., 2016).

Salt stress negatively regulates different developmental phase changes. In particular, seed germination (embryonic to the vegetative) and floral transition (vegetative to reproductive) are also promoted by GA. Precisely, the interplay between GA and the antagonistic phytohormone ABA tightly controls seed germination to ensure successful seedling establishment. The biosynthesis of ABA increases during embryo development to induce seed dormancy and prevent premature germination in the siliques. Likewise, ABA accumulates in response to drought and salinity to avoid seedling development under adverse conditions (Shu et al., 2018). Contrarily, in favorable conditions ABA content decreases rapidly after seed imbibition and the biosynthesis of GA increases to promote testa rupture followed by radicle protrusion from the seed coat (Shu et al., 2018). Similar to *rav1* and *rav1-like* mutants (Fu et al., 2014), also *tem* mutants showed promoted germination in salt stress conditions, suggesting that increased endogenous GA levels could compensate salt-induced inhibition of germination. Also, exogenous GA application exerts a stress-alleviating effect on radicle protrusion.

In addition, soil salinity severely affects vegetative growth of seedlings, resulting in a reduction of plant biomass. Increasing salt concentration inhibits the development of green organs and promotes leaf curling of wild type and mutant plants. However, *tem* mutants showed a significant although moderate higher fresh weight than wild types at mild salt stress conditions.

Besides negative effects of salinity on seedling establishment and general growth, salt stress also delays the floral transition in a dose dependent manner (Zhang et al., 2011), either by activating the floral repressor *FLC* or repressing the floral activator *LFY* (Li et al., 2007). Strikingly, *tem* mutants showed anticipated floral transition regardless of the treatment, indicating that TEMs might integrate floral transition and salt sensitivity at the molecular level. Actually, the salt-induced downregulation of *FT* and *TSF* in *tem* mutants resulted in levels above the threshold required to induce flowering.

Altogether, we propose a role for TEMs in the integration of plant development and salt stress response by regulating the accumulation of GAs and the activation of floral integrators. We hypothesise that a faster life cycle (as a consequence of shortened vegetative phase and early flowering) allows *tem* mutants to overcome growth inhibition triggered by salt. Vegetative growth restraint associated to early flowering could also be an advantage under adverse environmental conditions. Indeed, *tem* adult plants could be less vulnerable to abiotic stress because of the

reduced surface of their rosettes. At maturity, *tem* mutants are considerably smaller than wild-type plants just because vegetative development ceased at the floral transition, a process that is anticipated in the mutant background and leads to the formation of fewer leaves at bolting.

Intriguingly, *TEM1-OE* plants are dwarf but exhibited precocious senescence and hypersensitivity in response to salinity. This might suggest that TEMs play additional roles in transcriptional reprogramming underlying salt stress responses independently of other genetic pathways regulating plant size.

TEMs regulate salinity-induced senescence mediated by JA

Senescence, a physiological process leading to leaf yellowing, is developmentally regulated by aging but it can also be prematurely induced in response to abiotic and biotic stresses through increased levels of endogenous phytohormones such as JA, ABA and ethylene (He et al., 2002).

In our studies, we observed that the progression of salt-induced senescence seemed to be delayed in *tem* plants, probably as a result of the down-regulation of JA biosynthetic genes and consequently reduced content of this senescence accelerating hormone. As supporting evidence, senescence-associated molecular markers such as *MKK9* and selected *SAGs* were activated upon salt treatment in wild-types but down-regulated in the mutant background. In accordance with previous reports, *MKK9* was induced by JA and transcribed in early senescent leaves, whereas *SAG12* in late senescent leaves (Zhou et al., 2009)**.** Indeed, plants over-expressing *MKK9* are hyper-sensitive to salt and display precocious senescence, whereas loss of function *mkk9* mutants are more tolerant to salt and display delayed senescence (Xu et al., 2008; Zhou et al., 2009).

Also, *SAG113/HAI1* is induced in response to salinity and drought (Singh et al 2016) and downregulated in *tem* mutant. Interestingly, SAG113/HAI1 negatively regulates the expression of genes encoding dehydrins and Late Embryogenesis Abundant proteins, and the production of osmolytes including proline at low water potential (Bhaskara et al., 2012). Therefore, we propose that TEMs play regulatory roles in leaf senescence by modulating the expression of JA biosynthetic genes and regulators of the osmotic component of abiotic stress response, likely in an ABA-independent manner (Xu et al., 2008; Zhou et al., 2009; Bhaskara et al., 2012; Nguyen and Cheong, 2018).

TEMs have a function in the response to salt-induced oxidative stress

According to molecular data, also metabolic studies corroborated the delayed senescence in mutant plants subjected to salt stress. Indeed, the analysis of senescence-associated physiological markers revealed that the total content of chlorophylls and carotenoids decreased more drastically in the wild-type than in the mutant. In addition to the degradation of these pigments, also oxidative stress directly correlates with decreased photosynthetic capacity. Indeed, dramatic production of ROS impairs PhotoSystems (PS) by damaging membrane integrity via peroxidation of membrane lipids (Apel and Hirt, 2004). Together with the regulation of osmotic and ionic homeostasis, additional strategies to preserve plants from abiotic stresses include the increased production of secondary metabolites involved in the protection of PSs against toxic ROS levels. Specifically, accumulation of molecules with antioxidant properties - such as vitamin E - prevents the propagation of lipid peroxidation caused by increased ROS levels in stress conditions. In agreement, previous studies demonstrated that plants depleted in vitamin E are more sensitive to salt-induced oxidative stress (Ellouzi et al., 2013).

To mitigate photo-oxidative damage and avoid over-excitation of PSs under stress conditions, plants reduce light harvesting mainly by regulating the expression of genes encoding Chlorophyll a/b Binding factors and components of Light-Harvesting Complexes. For instance, ELIP2 hampers photo-oxidative stress induced by adverse external conditions by blocking accumulation of free chlorophyll. In this way, ELIP2 negatively regulates Chlorophyll synthesis as it sequesters available units for the assembly of pigment-binding proteins for photosynthesis (Tzvetkova-Chevolleau et al., 2007).

In our studies, we observed accumulation of α-tocopherol and increased levels of *ELIP2* in *tem* plants in response to salinity, suggesting a mitigation of photo-oxidative stress in the mutant. In summary, *tem* plants cope better with salt stress than wild-type plants due to reduced ROS accumulation, slower breakdown of pigments, and increased production of molecules with antioxidant properties (α-tocopherol and ELIP2). As photosynthesis is the major energy source for plant growth and is extremely sensitive to abiotic stresses, a fast adaptation of the plant to the environment is essential to deal with fluctuating conditions.

Although the direct binding of TEMs to putative targets goes beyond the scope of this article, a preliminary analysis of the regulatory regions of candidate genes revealed the presence of RAV consensus sequence in the promoters of TMT1/VTE4 and At5g49760 (Figure S10). The latter encodes a Leucine Rich Repeat receptor Kinase recently described as Hydrogen Peroxide sensor mediating the activation of Ca^{2+} channels in response to environmental stresses (Wu et al., 2020).

Future work could focus on deciphering the molecular mechanisms acting downstream of RAV factors. Actually, the understanding of different genetic factors controlling adaptive growth and photo-protection is crucial to develop plant varieties with increased climate resilience. This is particularly true for crops, where high biomass accumulation and yield are the major agronomic traits to be improved despite limitation of natural resources and agro-ecosystems.

Conclusions

Our studies have elucidated how TEM transcription factors regulate adaptation to environmental adversity such as increasing soil salinity. We specifically analyzed the effect of salt stress on different developmental stages and found that *tem1 tem2* plants perform better than the wild types when exposed to mild and high salt stress. We propose that TEMs act as hub in general mechanisms to integrate developmental programs and salt stress response by regulating the content of hormones with signaling function in adaptive growth (Figure 7). Actually, TEMs negatively regulate plant development and promote salinity-induced senescence by modifying gene expression of components of different signaling cascades (Figure 7). As previously suggested (Fu et al., 2014a), tolerance to salt might be obtained by reducing the expression of *RAV* genes or exploiting genetic variability at *RAV* loci. Therefore, these results provide new strategies to modulate plant development in response to adverse environmental conditions, which are major limiting factors for crop production.

EXPERIMENTAL PROCEDURES

Plant material and stress treatments

The wild-type plant used in this study was *Arabidopsis thaliana* ecotype Columbia 0 (Col-0). *TEM1* over-expressing (*TEM1-OE*) lines and *tem1 tem2* loss of function mutants in Col-0 background were described previously. Plants were grown in controlled conditions in LD (16 hours light/ 8 hours dark) or SD (8 hours light/ 16 hours dark) chambers at 22°C.

For molecular and phenotypic analyses during the juvenile phase, seeds were surface sterilized, cold treated at 4°C for three days in darkness and sown on plate containing half-strength Murashige and Skoog (MS) medium and 0.8% plant agar. Two days after imbibition, germinating seeds were divided into groups and transferred to different plates. Salt treatment was carried out by supplementing the medium with 50 and 100 mM NaCl, and GA treatment with 1 μM GA_3.

For molecular and phenotypic analyses at floral transition and during the adult phase, wild-type and *tem1 tem2* mutant seeds were sown on soil pots. Three Days After Germination (DAG), seedlings were divided into three groups and watered twice a week until maturity. Control group was irrigated with water, whereas mild and high salt stresses groups were irrigated with water containing 50 mM NaCl (low concentration) and 100 mM NaCl (medium concentration) respectively, to simulate natural salinity increase.

RNA-sequencing and analysis of Differentially Expressed Genes

High-throughput Sequencing of wild-type and *TEM1-OE* plants was done independently 2 times, and each sample was pooled from twenty 10-days old plants grown at LD 22ºC and collected at ZT18. Total RNA was extracted with PureLink™ RNA Mini Kit (Invitrogen), treated with TURBO DNA-*free*™ Kit (Invitrogen), and used for the construction of RNA-Seq cDNA libraries, following standard Illumina protocols. Sequencing was performed on Illumina HiSeq2500 instrument. Base calls were performed with RTA 1.13.48.0 followed by conversion to FASTQ with bcl2fastq 1.8.4. QC check was performed on the raw sequencing data, removing low-quality portions. The minimum length established was 35 and the quality score 25. FastQC was performed before and after the trimming. High-quality reads were mapped to the *Arabidopsis thaliana* reference genome (TAIR10) using Spliced Transcripts Alignment to Reference (STAR) software (v2.4.0). STAR alignment files were used as input for Cuffdiff (v2.2.1) together with the TAIR 10 annotation file to calculate gene expression values. Differentially Expressed Genes (DEGs) in gain of function mutant compared to wt were identified based on the criteria of fold change ≥2. Gene Ontology (GO) Analysis of DEGs was performed using AgriGO v2.0 and g:Profiler (http://systemsbiology.cau.edu.cn/agriGOv2/, https://biit.cs.ut.ee/gprofiler/gost). Top 10 GO-terms were used for generating GO chart. Locus identifiers of Araport were converted into UniProt KB using https://www.uniprot.org/uploadlists/, and UniProt identifiers to KO using https://www.genome.jp/kegg/tool/conv_id.html. Finally, KEGG pathway mapping was perfomed using https://www.genome.jp/kegg/tool/map_pathway1.html

Hormone analysis

For each genotype, plants were collected 5 days after bolting and the content of JA was measured as previously described (Matías-Hernandez et al., 2016).

Expression analysis

For each genotype and condition, pools of 100 developing seeds were collected two days after imbibition and pools of 20 plants were collected at different developmental stages (7, 14 and 21 Days After Germination). All samples were harvested at ZT12, based on diurnal oscillation of *TEM* genes and interesting candidates. Total RNA was extracted with Maxwell RSC Plant RNA kit (Promega) including an on-column DNase I digestion. After quality control, 1 μg of total RNA was used for first-strand cDNA synthesis by Superscript III Reverse Transcriptase (Invitrogen), and used for quantitative real-time RT-PCR on Light Cycler 480 II with SYBR Green I master mix (Roche). Three biological replicates with three technical replicates each were used for all analyses. ΔCt-values were calculated by subtracting Ct-values of the target gene from the Ct-value of the constitutively expressed *eIF4* gene. Relative expression levels were calculated as 2-ΔΔCt method, and the statistical significance was evaluated by unpaired t-test analysis. Primers used for expression analysis are reported in Table S3.

Phenotypic analyses

For germination assay, sterilized seeds were sown on MS medium and seedling grown in plates placed vertically in LD chambers. After imbibition, emergence of radicle through the seed coat was scored daily (up to 120 hours after imbibition) using stereomicroscope. The germination rate was calculated as percentage of seeds (out of 100 seeds) with protruded radicles for each time-point and reported as mean value of three biological replicates with Standard Deviation for each genotype and condition.

For seedling growth, pictures of 40 one-week-old seedling and 10 two-week-old plants were taken and processed using Image-J software to measure primary root length, cotyledon diameter and rosette area for each genotype and condition. For biomass accumulation, fresh weight of 20 two-week-old seedlings was measured and reported as mean value of three biological replicates with Standard Deviation for each genotype and condition.

For flowering time, 20–25 seeds per genotype and treatment were used and experiments were performed on soil-grown plants three times with similar results. Data are reported as number of rosette leaves formed and days to bolting of individual plants, with mean value of the pool.

For mortality, the percentage of dead plants out of 20 plants each genotype and condition was measured four weeks after germination. Experiments were performed on soil-grown plants two times with same results.

Physiological analyses

The accumulation of hydrogen peroxide was detected by staining whole 4-weeks-old Arabidopsis plants with 3,3'-diaminobenzidine (DAB) using an improved DAB staining method (Daudi and O'Brien, 2012).

The analysis of photosynthetic pigments and anti-oxidants was performed using **r**osette leaves dissected from 4-weeks-old plants, ground in liquid nitrogen and lyophilized. Secondary metabolites were extracted and analysed by High Performance Liquid Chromatography (HPLC, Agilent technology) as previously described (Rodriguez-Concepcion, 2004) using the non-plant carotenoid canthaxanthin as internal standard.

Chlorophyll fluorescence was measured in 5 weeks-old *Arabidopsis* plants with the MAXI version of the IMAGING-PAM *M*-Series fluorometer according to the manufacturer *(*Walz). After 30 minutes of dark adaptation, the kinetics of chlorophyll fluorescence in whole rosettes were monitored by measuring F_0 in the dark and Fm with initial Saturation pulse, Fm' and F' during a series of SAT-pulses of Actinic Light every 60 seconds for 10 minutes, followed by 30 minutes of dark relaxation. Values of Non Photochemical Quenching (NPQ) were calculated for each genotype and condition. These values indicate PSII performance of plants in response to salt stress.

In silico analysis of regulatory regions

Additional putative direct targets of TEMs were obtained by overlapping DEGs with a previously obtained list of genes containing RAV binding sites (Osnato et al., 2012). Regulatory regions of selected genes were analysed using Athamap (http://www.athamap.de/search_gene.php).

Statistical Analyses

Unpaired t-tests and One-way ANOVA analyses were conducted using GraphPad Prism7.00. Values are represented as means +/- Standard Deviation, with significant differences at $P<0.05$. All statistical analyses can be found in Table S4.

Accession Numbers

TEM1 (At1g25560), TEM2 (AT1G68840), FT (AT1G65480), TSF (AT4G20370), GA3OX1 (AT1G15550), SOC1 (AT2G45660); AOC1 (AT3G25760), AOC2 (AT3G25770), LOX2

(AT3G45140), LOX3 (AT1G17420), LOX4 (AT1G72120), MKK9 (AT1G73500), HAI1 (AT5G59220), SAG12 (AT5G45890), G-TMT1 (AT1G64970), ELIP2 (AT4G14690).

DATA AVAILABILITY STATEMENT

The RNA-Seq data discussed in this article have been deposited in NCBI´s Gene Expression Omnibus and are accessible through GEO Series accession number GSE148142 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE148142). Materials and data are available upon request to the corresponding author.

ACKNOWLEDGEMENTS

We thank Luca Morelli for advice with PAM-imaging and to our funders: MINECO-MICIU/FEDER (BFU2015-64409-P, PCG2018-095804-B-I00) to S.P., CERCA Programme/Generalitat de Catalunya (2017 SGR 718) and "Severo Ochoa Programme for Centres of Excellence in R&D" 2016-2019 (SEV-2015-0533) to CRAG. Work in J.L.R. laboratory was supported by MINECO/FEDER grant BFU2014-58289-P. A.E.A.-J. and U.C. performed this work within the frame of a Ph.D Program of the Universitat Autònoma de Barcelona with an Investigator Training Program PhD fellowship from Generalitat de Catalunya to A.E.A.-J. and a FPI fellowship from Spanish Ministry to U.C**.** Sequentia Biotech SL (Barcelona, Spain) helped with RNA-Seq analysis.

AUTHOR CONTRIBUTIONS

M.O. and S.P. planned experimental strategies and wrote the manuscript with inputs from other authors. M.O., U.C. and J.S. performed most of the experiments. L.M.-H. and A.E.A.-J. performed experiments for RNA-sequencing and quantification of hormone content. J.L.R. analysed RNA-sequencing. M.R.R.-G. and M.R.-C. helped with quantification of pigments and analysis of Photosynthetic efficiency. The authors declare that there is no conflict of interest.

CONFLICT OF INTEREST

The authors declare that they do not have competing interests.

FUNDING

This work was supported by MINECO-MICIU/FEDER (BFU2015-64409-P, PCG2018-095804-B-I00) to SP and (BFU2014-58289-P) to JLR, CERCA Programme/Generalitat de Catalunya (2017 SGR 718) and "Severo Ochoa Programme for Centres of Excellence in R&D" 2016-2019 (SEV-2015-0533) to CRAG. A.E.A.-J. and U.C. performed this work within the frame of a Ph.D Program of the Universitat Autònoma de Barcelona with an Investigator Training Program PhD fellowship from Generalitat de Catalunya to A.E.A.-J. and a FPI fellowship from Spanish Ministry to U.C.

SUPPORTING INFORMATION

Figure S1: Gene-set Enrichment Analysis for biological processes of Differentially Expressed Genes in *TEM1-OE.*

Figure S2: JA content and mapping of DEGs to KEGG Database.

Figure S3: Effect of salinity on the floral transition under SD and LD conditions.

Figure S4: Expression analysis of regulators of the floral transition.

Figure S5: Effect of salinity on wild-type and *tem* seed germination.

Figure S6: Effect of salinity on wild-type and *tem* seedling growth one week after germination.

Figure S7: Effect of salinity on wild-type and *tem* seedling growth two weeks after germination.

Figure S8: Expression analysis of components of the JA signaling pathway.

Figure S9: Analysis of photoprotection and senescence in response to salt stress.

Figure S10: In silico promoter analysis of putative downstream targets of TEMs.

Table S1: Content of carotenoids, chlorophylls and tocopherols in *tem1tem2* after different salt treatments.

Table S2: Time point measurement of IMAGING-PAM fluorometry.

Table S3: List of primer sequences.

Table S4: Statistical analyses.

Dataset I: Differential expressed genes in *TEM1-OE* plants compared to wild types (excel file).

REFERENCES

Achard, P., Cheng, H., De Grauwe, L., Decat, J., Schoutteten, H., Moritz, T., Van Der Straeten, D., Peng, J. and Harberd, N.P. (2006) Integration of plant responses to environmentally activated phytohormonal signals. *Science*, **311**, 91-94.

Achard, P., Renou, J.P., Berthomé, R., Harberd, N.P. and Genschik, P. (2008) Plant DELLAs Restrain Growth and Promote Survival of Adversity by Reducing the Levels of Reactive Oxygen Species. *Curr Biol*. **18**, 656-660.

Apel, K. and Hirt, H. (2004) REACTIVE OXYGEN SPECIES: Metabolism, Oxidative Stress, and Signal Transduction. *Annu Rev Plant Biol*. **55**, 373-399.

Apse, M.P., Aharon, G.S., Snedden, W.A. and Blumwald, E. (1999) Salt tolerance conferred by overexpression of a vacuolar Na+/H+ antiport in Arabidopsis. *Science*, **285**, 1256-1258.

Baier, M., Kandlbinder, A., Golldack, D. and Dietz, K.J. (2005) Oxidative stress and ozone: Perception, signalling and response. *Plant, Cell Environ*. **28**, 1012-1020.

Bergmüller, E., Porfirova, S. and Dörmann, P. (2003) Characterization of an Arabidopsis mutant deficient in γ-tocopherol methyltransferase. *Plant Mol Biol*. **52**, 1181-1190.

Bhaskara, G.B., Nguyen, T.T. and Verslues, P.E. (2012) Unique drought resistance functions of the highly ABA-induced clade a protein phosphatase 2Cs. *Plant Physiol*. **160**, 379-395.

Castillejo, C. and Pelaz, S. (2008) The Balance between CONSTANS and TEMPRANILLO Activities Determines FT Expression to Trigger Flowering. *Curr Biol*. **18**, 1338-1343.

Chini, A., Fonseca, S., Fernández, G., Adie, B., Chico, J.M., Lorenzo, O., García-Casado, G., López-Vidriero, I., Lozano, F.M., Ponce, M.R., et al (2007) The JAZ family of repressors is the missing link in jasmonate signalling. *Nature*, **448**, 666-671.

Daudi, A. and O'Brien, J.A. (2012) Detection of hydrogen peroxide by DAB staining in *Arabidopsis* leaves. *Bio Protoc*. **2**(18):e263.

Deinlein, U., Stephan, A.B., Horie, T., Luo, W., Xu, G. and Schroeder, J.I. (2014) Plant salt-tolerance mechanisms. *Trends Plant Sci*. **19**, 371-379.

Duan, Y.B., Li, J., Qin, R.Y., Xu, R.F., Li, H., Yang, Y.C., Ma, H., Li, L., Wei, P.C. and Yang, J.B. (2016) Identification of a regulatory element responsible for salt induction of rice OsRAV2 through ex situ and in situ promoter analysis. *Plant Mol Biol*. **90**, 49–62.

Ellouzi, H., Hamed, K.B., Cela, J., Müller, M., Abdelly, C. and Munné-Bosch, S. (2013) Increased sensitivity to salt stress in tocopherol-deficient Arabidopsis mutants growing in a hydroponic system. *Plant Signal Behav*. **8**(2):e23136.

Fu, M., Kang, H.K., Son, S.-H., Kim, S.-K. and Nam, K.H. (2014) A Subset of RAV Transcription Factors Modulates Drought and Salt Stress Responses ABA-Independently in Arabidopsis. *Plant Cell Physiol*. **55**, 1892-1904.

Gepstein, S., Sabehi, G., Carp, M.J., Hajouj, T., Nesher, M.F.O., Yariv, I., Dor, C. and

Bassani, M. (2003) Large-scale identification of leaf senescence-associated genes. *Plant J*. **36**, 629-642.

Golldack, D., Li, C., Mohan, H. and Probst, N. (2014) Tolerance to drought and salt stress in plants: Unraveling the signaling networks. *Front Plant Sci*. **5**:e151.

Hasegawa, M., Bressan, R. and Pardo, J.M. (2000) The dawn of plant salt tolerance genetics. *Trends Plant Sci*. **5**, 317-319.

He, Y., Fukushige, H., Hildebrand, D.F. and Gan, S. (2002) Evidence supporting a role of jasmonic acid in Arabidopsis leaf senescence. *Plant Physiol*. **128**, 876-884.

Hutin, C., Nussaume, L., Moise, N., Moya, I., Kloppstech, K. and Havaux, M. (2003) Early light-induced proteins protect Arabidopsis from photooxidative stress. *Proc Natl Acad Sci USA*. **100**, 4921-4926.

James, M., Poret, M., Masclaux-Daubresse, C., Marmagne, A., Coquet, L., Jouenne, T., Chan, P., Trouverie, J. and Etienne, P. (2018) SAG12, a major cysteine protease involved in nitrogen allocation during senescence for seed production in Arabidopsis thaliana. *Plant Cell Physiol*. **59**, 2052-2063.

Jiang, Y. and Deyholos, M.K. (2009) Functional characterization of Arabidopsis NaCl-inducible WRKY25 and WRKY33 transcription factors in abiotic stresses. *Plant Mol Biol*. **69**, 91-105.

Kim, S.G., Lee, A.K., Yoon, H.K. and Park, C.M. (2008) A membrane-bound NAC transcription factor NTL8 regulates gibberellic acid-mediated salt signaling in *Arabidopsis* seed germination. *Plant J*. **55**, 77-88.

Kuai, B., Chen, J. and Hörtensteiner, S. (2018) The biochemistry and molecular biology of chlorophyll breakdown. *J Exp Bot*. **69**, 751-767.

Li, K., Wang, Y., Han, C., Zhang, W., Jia, H. and Li, X. (2007) GA signaling and CO/FT regulatory module mediate salt-induced late flowering in Arabidopsis thaliana. *Plant Growth Regul*. **53**, 195-206.

Li, X.J., Li, M., Zhou, Y., Hu, S., Hu, R., Chen, Y. and Li, X.B. (2015) Overexpression of cotton RAV1 gene in Arabidopsis confers transgenic plants high salinity and drought sensitivitye0118056. *PLoS One*. **10**(2), e0118056.

Li, Z., Zhao, Y., Liu, X., Peng, J., Guo, H. and Luo, J. (2014) LSD 2.0: An update of the leaf senescence database. *Nucleic Acids Res*. **42**: D1200-1205.

Lim, P.O., Kim, H.J. and Gil Nam, H. (2007) Leaf Senescence. *Annu Rev Plant Biol*. **58**, 115-136.

Lim, P.O., Woo, H.R. and Nam, H.G. (2003) Molecular genetics of leaf senescence in Arabidopsis. *Trends Plant Sci*. **8**, 272-278.

Liu, J., Ishitani, M., Halfter, U., Kim, C.S. and Zhu, J.K. (2000) The Arabidopsis thaliana SOS2 gene encodes a protein kinase that is required for salt tolerance. *Proc Natl Acad Sci USA*. **97**, 3730-3734.

Liu, X., Li, Z., Jiang, Z., Zhao, Y., Peng, J., Jin, J., Guo, H. and Luo, J. (2011) LSD: A leaf senescence database. *Nucleic Acids Res*. **39**, D1103-1107.

Marín-González, E., Matías-Hernández, L., Aguilar-Jaramillo, A.E., Lee, J.H., Ahn, J.H., Suárez-López, P. and Pelaz, S. (2015) SHORT VEGETATIVE PHASE Up-Regulates TEMPRANILLO2 Floral Repressor at Low Ambient Temperatures. *Plant Physiol*. **169**, 1214-1224.

Matías-Hernández, L., Aguilar-Jaramillo, A.E., Osnato, M., Weinstain, R., Shani, E., Suárez-López, P. and Pelaz, S. (2016a) TEMPRANILLO reveals the mesophyll as crucial for epidermal trichome formation. *Plant Physiol*. **170**, 1624-1639.

Matile, P., Hörtensteiner, S., Thomas, H. and Kräutler, B. (1996) Chlorophyll breakdown in senescent leaves. *Plant Physiol*. **112**, 1403-1409.

Neill, S., Desikan, R. and Hancock, J. (2002) Hydrogen peroxide signalling. *Curr Opin Plant Biol*. **5**, 388-395.

Nguyen, N.H. and Cheong, J.J. (2018) AtMYB44 interacts with TOPLESS-RELATED corepressors to suppress protein phosphatase 2C gene transcription. *Biochem Biophys Res Commun*. **507**, 437-442.

Noh, Y.S. and Amasino, R.M. (1999) Identification of a promoter region responsible for the senescence-specific expression of SAG12. *Plant Mol Biol*. **41**, 181-194.

Osnato, M., Castillejo, C., Matías-Hernández, L. and Pelaz, S. (2012) TEMPRANILLO genes link photoperiod and gibberellin pathways to control flowering in Arabidopsis. *Nat Commun*. **3**, e808.

Otegui, M.S., Noh, Y.S., Martínez, D.E., Vila Petroff, M.G., Staehelin, L.A., Amasino, R.M. and Guiamet, J.J. (2005) Senescence-associated vacuoles with intense proteolytic activity develop in leaves of Arabidopsis and soybean. *Plant J*. **41**, 831-844.

Park, H.J., Kim, W.Y. and Yun, D.J. (2016) A new insight of salt stress signaling in plant. *Mol Cells*, **39**, 447-459.

Putterill, J., Robson, F., Lee, K., Simon, R. and Coupland, G. (1995) The CONSTANS gene of

arabidopsis promotes flowering and encodes a protein showing similarities to zinc finger transcription factors. *Cell*, **80**, 847-857.

Qiu, Q.S., Guo, Y., Dietrich, M.A., Schumaker, K.S. and Zhu, J.K. (2002) Regulation of SOS1, a plasma membrane Na+/H+ exchanger in Arabidopsis thaliana, by SOS2 and SOS3. *Proc Natl Acad Sci USA*. **99**, 8436-8441.

Rodríguez-Concepción, M. (2004) The MEP pathway: a new target for the development of herbicides, antibiotics and antimalarial drugs. *Curr Pharm Des*. **19**, 2391-2400.

Ruban, A.V. (2016) Nonphotochemical chlorophyll fluorescence quenching: Mechanism and effectiveness in protecting plants from photodamage. *Plant Physiol*. **170**, 1903-1916.

Samach, A., Onouchi, H., Gold, S.E., Ditta, G.S., Schwarz-Sommer, Z., Yanofsky, M.F. and Coupland, G. (2000) Distinct roles of constans target genes in reproductive development of Arabidopsis. *Science*, **5471**, 1613-1616.

Semchuk, N.M., Lushchak, O.V., Falk, J., Krupinska, K. and Lushchak, V.I. (2009) Inactivation of genes, encoding tocopherol biosynthetic pathway enzymes, results in oxidative stress in outdoor grown Arabidopsis thaliana. *Plant Physiol Biochem*. **47**, 384-390.

Shavrukov, Y. (2013) Salt stress or salt shock: Which genes are we studying? *J Exp Bot*. **64**, 119-127.

Shi, H., Ishitani, M., Kim, C. and Zhu, J.K. (2000) The Arabidopsis thaliana salt tolerance gene SOS1 encodes a putative Na+/H+ antiporter. *Proc Natl Acad Sci USA*. **67**, 6896-6901.

Shi, H., Lee, B.H., Wu, S.J. and Zhu, J.K. (2003) Overexpression of a plasma membrane Na+/H+ antiporter gene improves salt tolerance in Arabidopsis thaliana. *Nat Biotechnol*. **21**, 81-85.

Shi, H., Quintero, F.J., Pardo, J.M. and Zhu, J.K. (2002) The putative plasma membrane NA+/H+ antiporter SOS1 controls long-distance NA+ transport in plants. *Plant Cell*, **14**, 465-477.

Shu, K., Zhou, W., Chen, F., Luo, X. and Yang, W. (2018) Abscisic acid and gibberellins antagonistically mediate plant development and abiotic stress responses. *Front Plant Sci*. **9**, e416.

Skubacz, A., Daszkowska-Golec, A. and Szarejko, I. (2016) The role and regulation of ABI5 (ABA-insensitive 5) in plant development, abiotic stress responses and phytohormone crosstalk. *Front Plant Sci*. **7**, e1884.

Sohn, K.H., Lee, S.C., Jung, H.W., Hong, J.K. and Hwang, B.K. (2006) Expression and

functional roles of the pepper pathogen-induced transcription factor RAV1 in bacterial disease resistance, and drought and salt stress tolerance. *Plant Mol Biol*. **61**, 897-915.

Székely, G., Ábrahám, E., Cséplo, Á., Rigó, G., Zsigmond, L., Csiszár, J., Ayaydin, F., Strizhov, N., Jásik, J., Schmelzer, E., et al (2008) Duplicated P5CS genes of Arabidopsis play distinct roles in stress regulation and developmental control of proline biosynthesis. *Plant J*. **53**, 11-28.

Tester, M. and Langridge, P. (2010) Breeding technologies to increase crop production in a changing world. *Science*, **327**, 818-822.

Tracy, F.E., Gilliham, M., Dodd, A.N., Webb, A.A.R. and Tester, M. (2008) NaCl-induced changes in cytosolic free Ca2+ in Arabidopsis thaliana are heterogeneous and modified by external ionic composition. *Plant, Cell Environ*. **31**, 1063-1073.

Tzvetkova-Chevolleau, T., Franck, F., Alawady, A.E., Dall'Osto, L., Carrière, F., Bassi, R., Grimm, B., Nussaume, L., Havaux, M. (2007) The light stress-induced protein ELIP2 is a regulator of chlorophyll synthesis in Arabidopsis thaliana. *Plant J*. **50**, 795-809.

Woo, H.R., Goh, C.H., Park, J.H., De La Serve, B.T., Kim, J.H., Park, Y.Il and Nam, H.G. (2002) Extended leaf longevity in the ore4-1 mutant of Arabidopsis with a reduced expression of a plastid ribosomal protein gene. *Plant J*. **31**, 331-340.

Woo, H.R., Kim, H.J., Lim, P.O. and Nam, H.G. (2019) Leaf Senescence: Systems and Dynamics Aspects. *Annu Rev Plant Biol*. **70**, 347-376.

Wu, F., Chi, Y., Jiang Z, Xu Y, Xie L, Huang F, Wan D, Ni J, Yuan F, Wu X, Zhang Y, Wang L, Ye R, Byeon B, Wang W, Zhang S, Sima M, Chen S, Zhu M, Pei J, Johnson DM, Zhu S, Cao X, Pei C, Zai Z, Liu Y, Liu T, Swift GB, Zhang W, Yu M, Hu Z, Siedow JN, Chen X, Pei ZM. (2020) Hydrogen peroxide sensor HPCA1 is an LRR receptor kinase in Arabidopsis. *Nature*, **578**, 577-581.

Xu, J., Li, Y., Wang, Y., Liu, H., Lei, L., Yang, H., Liu, G. and Ren, D. (2008) Activation of MAPK kinase 9 induces ethylene and camalexin biosynthesis and enhances sensitivity to salt stress in Arabidopsis. *J Biol Chem*. **283**, 26996-27006.

Zhang, Z., Zhang, S., Zhang, Y., Wang, X., Li, D., Li, Q., Yue, M., Li, Q., Zhang, Y.E., Xu, Y., et al (2011) Arabidopsis floral initiator SKB1 confers high salt tolerance by regulating transcription and pre-mRNA splicing through altering histone H4R3 and small nuclear ribonucleoprotein LSM4 methylation. *Plant Cell*, **23**, 396-411.

Zhao, S.P., Xu, Z.S., Zheng, W.J., Zhao, W., Wang, Y.X., Yu, T.F., Chen, M., Zhou, Y.B.,

Min, D.H. and Ma, Y.Z., et al (2017) Genome-wide analysis of the rav family in soybean and functional identification of GmRAV-03 involvement in salt and drought stresses and exogenous ABA treatment. *Front Plant Sci*. **8**, e905.

Zhou, C., Cai, Z., Guo, Y. and Gan, S. (2009) An Arabidopsis mitogen-activated protein kinase cascade, MKK9-MPK6, plays a role in leaf senescence. *Plant Physiol*. **150**, 167-177.

FIGURE LEGENDS

Figure 1. GO analysis of Differentially Expressed Genes in *TEM1-OE*.
(a) Enrichment of categories related to hormone signaling based on GO analysis of genes downregulated in *TEM1-OE.* (b) Enrichment of categories related to water deprivation based on GO analysis of genes up-regulated in *TEM1-OE.* Gene-set enrichment analysis (GSEA) for biological processes of ranked gene list (Fold Change>2) of DEGs was performed using gProfiler. The ten most significant GO-terms are shown.

Figure 2. Salt stress response of plants mis-expressing *TEM* genes.
(a) Line charts showing the negative effect of increasing soil salinity on the survival rate of wild type, *tem1 tem2* and *TEM1-OE* plants, grown for 4 weeks under LD. (b) Top views of adult wild-type and *tem1 tem2* plants subjected to mild (orange) and high (red) salt stress, compared to control (white). (c) Left, top views of adult *TEM1-OE* plants showing hypersensitivity to salt treatment. Right, alterations of *TEM1-OE* rosette color assessed by using Image Color Picker. Color codes of the different samples are indicated. (d) and (e) Side views of wild-type and *tem1 tem2* plants grown for 5 weeks under LD showing negative effect of increasing soil salinity on reproductive growth.

Figure 3. Molecular and phenotypic analysis of flowering time in response to salt stress
(a) Inhibitory effect of increasing soil salinity on growth of wild-type and *tem1 tem2* plants grown for 3 weeks under LD. (b) Scatter plots showing flowering time under LD in response to mild (orange) and high (red) salt stress, scored as number of days to bolting and number of rosette leaves formed. Shown are individual data points and mean values for wild-type and *tem1 tem2.* Kruskal Wallis one-way ANOVA followed by Dunn's multiple comparisons test was used to assess significant differences between treatments and genotypes, with *P < 0.0033, **P < 0.0002,

***P < 0.0001. (c) and (d) Expression analysis of positive regulators of the floral transition in wild-type and *tem1 tem2* seedlings grown under LD for one week in controlled versus salt stress conditions. (c) RT-qPCR of the florigens *FT* and *TSF*. (d) RT-qPCR of *GA3OX1* and the floral integrator *SOC1*. One-way ANOVA followed by Tukey's multiple comparisons test was used to assess the statistical significance of differences between treatments and genotypes with *P < 0.0033, **P < 0.0002, ***P < 0.0001. For phenotypic and molecular analyses, three independent replicates gave similar results; one was chosen as representative and the others are reported in Figures S3 and S4.

Figure 4. Analysis of senescence associated molecular markers in response to salt stress.
(a) and (b) Downregulation of JA biosynthetic genes *AOC1-AOC2* and *LOX2-LOX3-LOX4* in *tem* mutant seedlings grown under LD for 1 week in all conditions. (c) RT-qPCR of *LOX2-LOX3-LOX4* in plants grown under LD for 2 weeks in controlled versus salt stress conditions. (d) and (e) downregulation of *MKK9* and *HAI1* in 2-week-old *tem* mutants grown under LD in response to salt stress. (f) Activation of *SAG12* in 3-week-old wild-type plants grown under LD in response to high salt stress. One-way ANOVA followed by Tukey's multiple comparisons test was used to assess the statistical significance of differences between treatments and genotypes with *P < 0.0033, **P < 0.0002, ***P < 0.0001. Independent replicates gave similar results; one was chosen as representative and the others are reported in Figures S8 and S9.

Figure 5. Analysis of ROS accumulation and photo-protection in plants undergoing salinity-induced senescence.
(a) DAB staining of H_2O_2 in wild-type, *tem* and *TEM1-OE* grown under LD for 4 weeks and irrigated with increasing salt concentration. (b) Upregulation of *gTMT1/VTE4* in stressed plants compared to controls after 2 and 3 weeks of salt treatment. (c) Accumulation of α-tocopherols in 4-week-old wild-type and *tem* plants grown under LD for 2 and 3 weeks. (d) Upregulation of *ELIP2* in salt-stressed *tem* plants grown under LD for 2 and 3 weeks. One-way ANOVA followed by Tukey's multiple comparisons test was used to assess the statistical significance of differences between treatments and genotypes with *P < 0.0033, **P < 0.0002, ***P < 0.0001. Independent replicates gave similar results; one was chosen as representative and the others are reported in Figures S9.

Figure 6. Analysis of senescence associated physiological markers and NPQ in response to salinity.

(a) Superimposed scatter plots showing the degradation of chlorophylls and carotenoids in response to salt stress, measured as total content of photosynthetic pigments in 4-weeks old wild-type (white) and *tem1 tem2* (green) plants grown under LD. (b) to (d) Superimposed scatter plots with connecting lines representing NPQ kinetic profiles of 5-weeks old wild-type (white) and *tem1 tem2* (green) plants grown under LD in controlled (b), mild (c) and high (d) salt stress conditions. NPQ was calculated as (Fo-Fm)/Fm at different time-points. White bar, induction and steady phase with actinic light; grey bar, relaxion phase in darkness. Two-tailed unpaired t-test was used to assess significant differences between the two genotypes, with *P < 0.0033, **P < 0.0002, ***P < 0.0001.

Figure 7. Effect of salinity on plant development in wild-type and *tem* plants.

Soil salinity affects different steps of the plant life cycle, especially successive developmental transitions such as embryonic to vegetative (germination) and vegetative to reproductive (floral transition) phase changes. In wild types, salt activates signaling pathways which enhance negative effects on growth partly mediated by the reduction in bioactive GA biosynthesis. In addition, salt extends the duration of the vegetative phase by maintaining low levels of floral activators and promotes senescence by damaging cell physiology. As TEMs mediate the negative regulation of plant development and positive regulation of leaf senescence, loss of function mutants tolerate salt stress because of a shorter life cycle and accumulation of molecules able to detoxify free radicals. The model was created in BioRender.com.

Dataset I. Genes differentially expressed in *TEM1-OE* plants.

(a)

Term name	Term ID	Padj	-log10(Padj)
Response to Jasmonic Acid	GO:0009753	7.113×10^{-4}	
Cellular response to endogenous stimulus	GO:0071495	6.345×10^{-3}	
Cellular response to hormone stimulus	GO:0032870	1.269×10^{-2}	
Response to oxygen-containing compound	GO:1901700	1.419×10^{-2}	
Hormone-mediated signalling pathway	GO:0009755	2.308×10^{-2}	
Cellular response to organic substance	GO:0071310	2.547×10^{-2}	
Defence response to insect	GO:0002213	3.332×10^{-2}	
Response to endogenous stimulus	GO:0009719	4.112×10^{-2}	
Response to organic substance	GO:0010033	4.243×10^{-2}	
Response to external biotic stimulus	GO:0043207	4.807×10^{-2}	

(b)

Term name	Term ID	Padj	-log10(Padj)
Response to water	GO:0009415	2.825×10^{-10}	
Response to water deprivation	GO:0009414	1.785×10^{-9}	
Response to inorganic substance	GO:0010035	8.600×10^{-7}	
Plant-type cell wall organization	GO:0009664	9.620×10^{-6}	
Cellular response to iron ion starvation	GO:0010106	1.093×10^{-5}	
Response to extracellular stimulus	GO:0009991	1.520×10^{-5}	
Response to acid chemical	GO:0001101	1.574×10^{-5}	
Response to osmotic stress	GO:0006970	2.830×10^{-5}	
Response to abiotic stimulus	GO:0009628	3.823×10^{-5}	
Response to nutrient levels	GO:0031667	7.901×10^{-5}	

Figure 1. GO analysis of Differentially Expressed Genes in *TEM1-OE*.
(a) Enrichment of categories related to hormone signaling based on GO analysis of genes downregulated in *TEM1-OE*. (b) Enrichment of categories related to water deprivation based on GO analysis of genes up-regulated in *TEM1-OE*. Gene-set enrichment analysis (GSEA) for biological processes of ranked gene list (Fold Change>2) of DEGs was performed using gProfiler. The ten most significant GO-terms are shown.

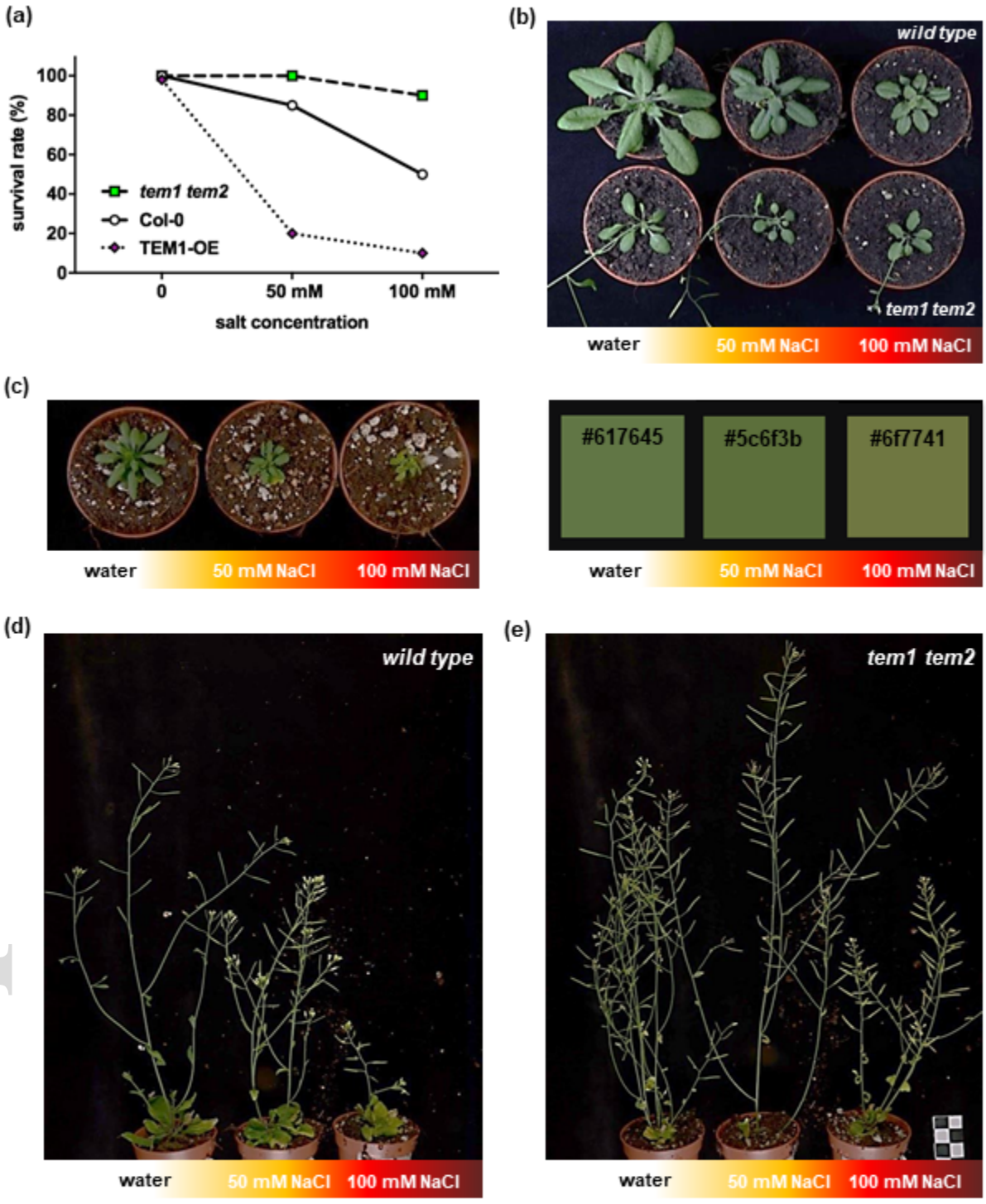

Figure 2. Salt stress response of plants mis-expressing *TEM* genes.
(a) Line charts showing the negative effect of increasing soil salinity on the survival rate of wild type, *tem1 tem2* and *TEM1-OE* plants, grown for 4 weeks under LD. (b) Top views of adult wild-type and *tem1 tem2* plants subjected to mild (orange) and high (red) salt stress, compared to control (white). (c) Left, top views of adult *TEM1-OE* plants showing hypersensitivity to salt treatment. Right, alterations of *TEM1-OE* rosette color assessed by using Image Color Picker. Color codes of the different samples are indicated. (d) and (e) Side views of wild-type and *tem1 tem2* plants grown for 5 weeks under LD showing negative effect of increasing soil salinity on reproductive growth.

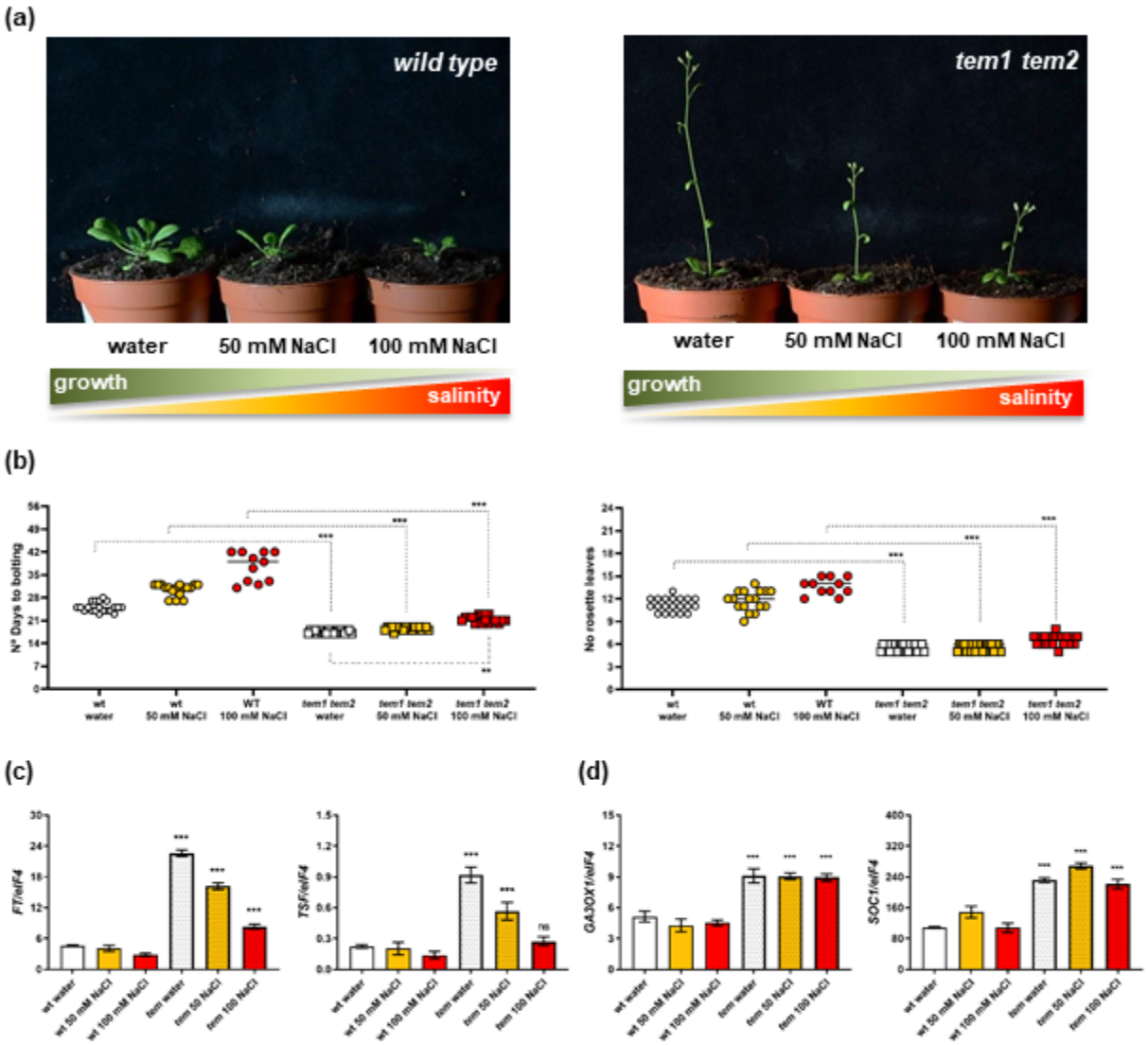

Figure 3. Molecular and phenotypic analysis of flowering time in response to salt stress
(a) Inhibitory effect of increasing soil salinity on growth of wild-type and *tem1 tem2* plants grown for 3 weeks under LD. (b) Scatter plots showing flowering time under LD in response to mild (orange) and high (red) salt stress, scored as number of days to bolting and number of rosette leaves formed. Shown are individual data points and mean values for wild-type and *tem1 tem2*. Kruskal Wallis one-way ANOVA followed by Dunn's multiple comparisons test was used to assess significant differences between treatments and genotypes, with *P < 0.0033, **P < 0.0002, ***P < 0.0001. (c) and (d) Expression analysis of positive regulators of the floral transition in wild-type and *tem1 tem2* seedlings grown under LD for one week in controlled versus salt stress conditions. (c) RT-qPCR of the florigens *FT* and *TSF*. (d) RT-qPCR of *GA3OX1* and the floral integrator *SOC1*. One-way ANOVA followed by Tukey's multiple comparisons test was used to assess the statistical significance of differences between treatments and genotypes with *P < 0.0033, **P < 0.0002, ***P < 0.0001. For phenotypic and molecular analyses, three independent replicates gave similar results; one was chosen as representative and the others are reported in Figures S3 and S4.

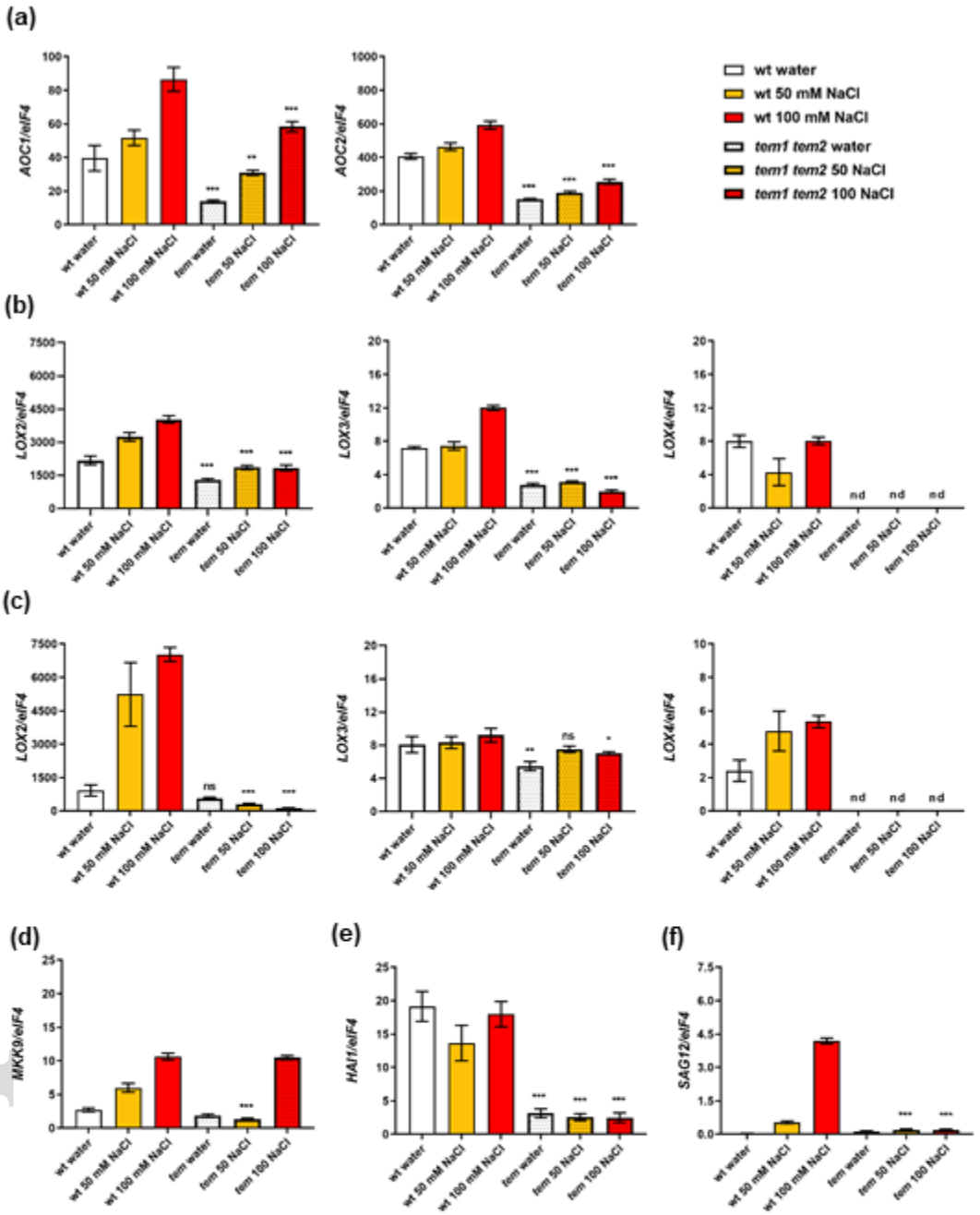

Figure 4. Analysis of senescence associated molecular markers in response to salt stress.
(a) and (b) Downregulation of JA biosynthetic genes *AOC1-AOC2* and *LOX2-LOX3-LOX4* in *tem* mutant seedlings grown under LD for 1 week in all conditions. (c) RT-qPCR of *LOX2-LOX3-LOX4* in plants grown under LD for 2 weeks in controlled versus salt stress conditions. (d) and (e) downregulation of *MKK9* and *HAI1* in 2-week-old *tem* mutants grown under LD in response to salt stress. (f) Activation of *SAG12* in 3-week-old wild-type plants grown under LD in response to high salt stress. One-way ANOVA followed by Tukey's multiple comparisons test was used to assess the statistical significance of differences between treatments and genotypes with *P < 0.0033, **P < 0.0002, ***P < 0.0001. Independent replicates gave similar results; one was chosen as representative and the others are reported in Figures S8 and S9.

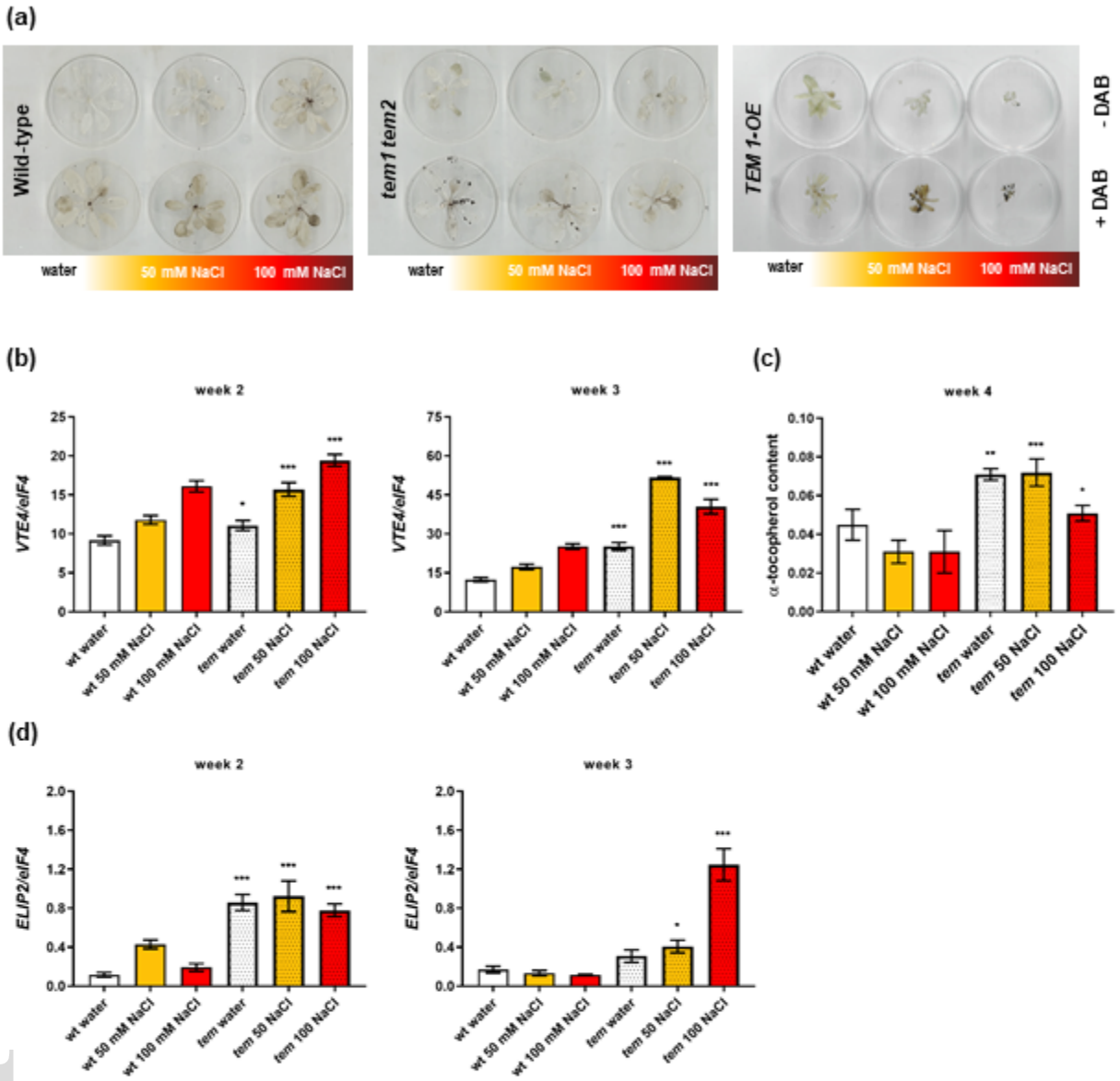

Figure 5. Analysis of ROS accumulation and photo-protection in plants undergoing salinity-induced senescence.
(a) From left to right, DAB staining of H_2O_2 in wild-type, *tem* and *TEM1-OE* plants grown under LD for 4 weeks and irrigated with increasing salt concentration. (b) Upregulation of *g-TMT1/VTE4* in stressed plants compared to controls after 2 and 3 weeks of salt treatment. (c) Accumulation of α-tocopherol in 4-week-old wild-type and *tem* plants grown under LD in controlled versus salt stress conditions. (d) Upregulation of *ELIP2* in salt-stressed *tem* plants grown under LD for 2 and 3 weeks. One-way ANOVA followed by Tukey's multiple comparisons test was used to assess the statistical significance of differences between treatments and genotypes with *P < 0.0033, **P < 0.0002, ***P < 0.0001. Independent replicates gave similar results; one was chosen as representative and the others are reported in Figure S9.

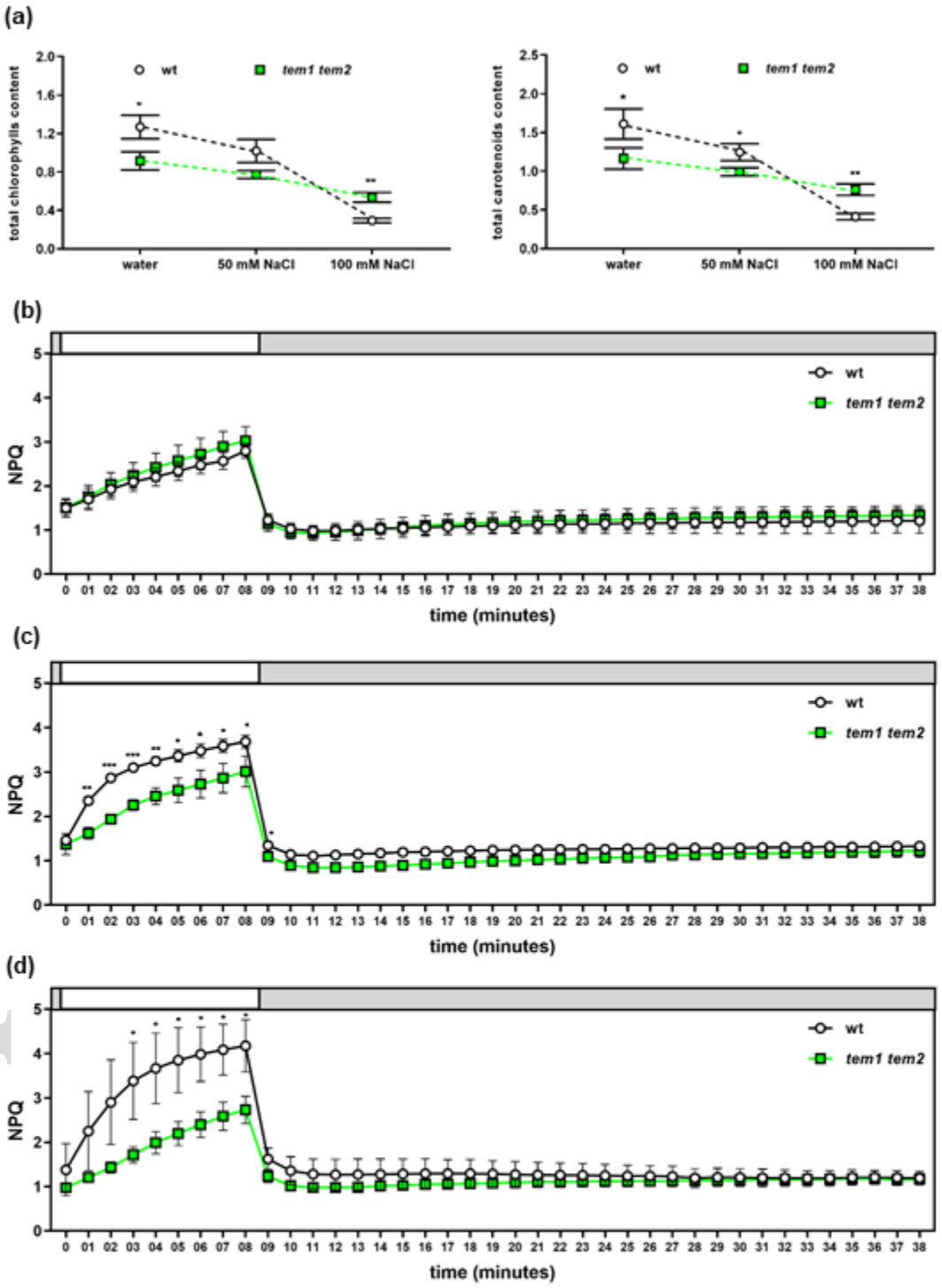

Figure 6. Analysis of senescence associated physiological markers and NPQ in response to salinity. (a) Superimposed scatter plots showing the degradation of chlorophylls and carotenoids in response to salt stress, measured as total content of photosynthetic pigments in 4-weeks old wild-type (white) and *tem1 tem2* (green) plants grown under LD. (b) to (d) Superimposed scatter plots with connecting lines representing NPQ kinetic profiles of 5-weeks old wild-type (white) and *tem1 tem2* (green) plants grown under LD in controlled (b), mild (c) and high (d) salt stress conditions. NPQ was calculated as (Fo-Fm)/Fm at different time-points. White bar, induction and steady phase with actinic light; grey bar, relaxion phase in darkness. Two-tailed unpaired t-test was used to assess significant differences between the two genotypes, with *P < 0.0033, **P < 0.0002, ***P < 0.0001.

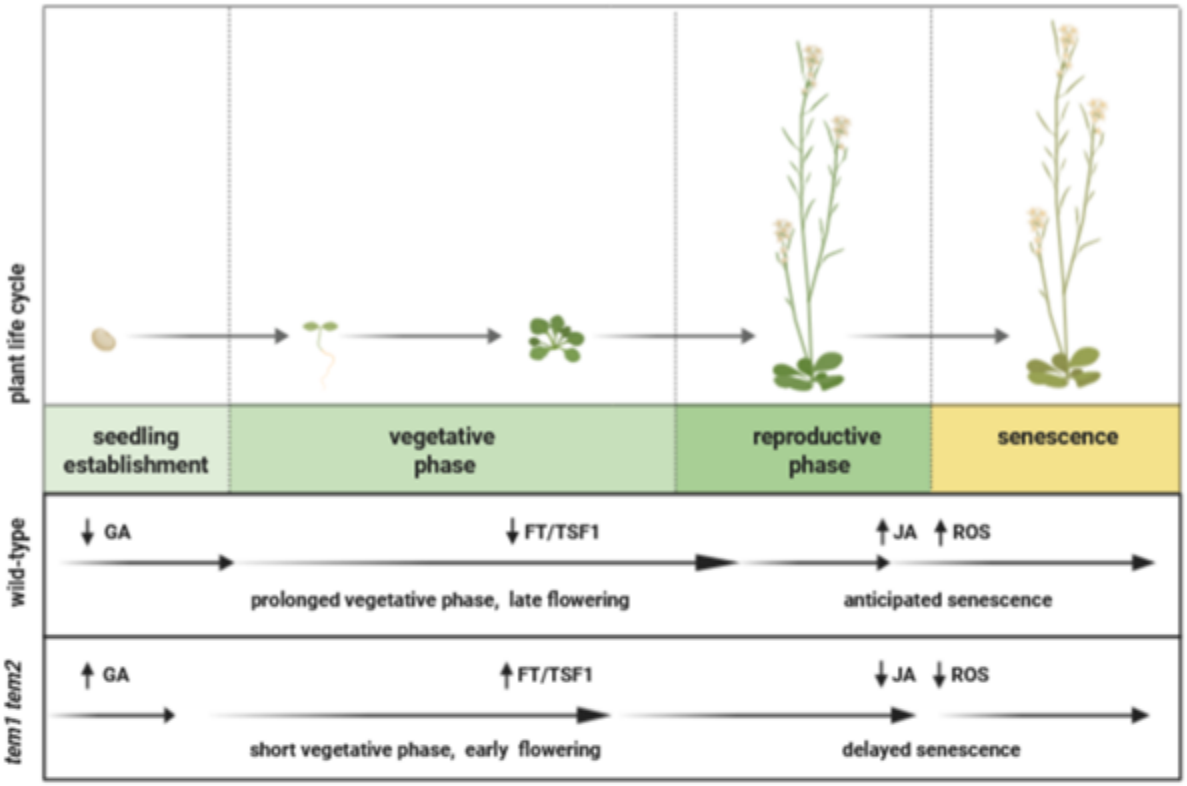

Figure 7. Effect of salinity on plant development in wild-type and *tem* plants.
Soil salinity affects different steps of the plant life cycle, especially successive developmental transitions such as embryonic to vegetative (germination) and vegetative to reproductive (floral transition) phase changes. In wild types, salt activates signaling pathways which enhance negative effects on growth partly mediated by the reduction in bioactive GA biosynthesis. In addition, salt extends the duration of the vegetative phase by maintaining low levels of floral activators and promotes senescence by damaging cell physiology. As TEMs mediate the negative regulation of plant development and positive regulation of leaf senescence, loss of function mutants tolerate salt stress because of a shorter life cycle and accumulation of molecules able to detoxify free radicals. The model was created in BioRender.com.