Soil salinity inhibits plant shade avoidance

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Summary

Global food production is set to keep increasing despite a predicted decrease in total arable land [1]. To achieve higher production, denser planting will be required on increasingly degraded soils. When grown in dense stands, crops elongate and raise their leaves in an effort to reach sunlight, a process termed shade-avoidance [2]. Shade is perceived by a reduction in the ratio of red (R) to (FR) light and results in the stabilisation of a class of transcription factors known as PHYTOCHROME INTERACTING FACTORs (PIFs) [3,4]. PIFs activate the expression of auxin biosynthesis genes [4,5] and enhance auxin sensitivity [6], which promotes cell wall loosening and drives elongation growth. Despite our molecular understanding of shade-induced growth, little is known about how this developmental programme is integrated with other environmental factors.

Here we demonstrate that low levels of NaCl in soil strongly impair the ability of plants to respond to shade. This block is dependent upon abscisic acid (ABA) signalling and the canonical ABA signalling pathway. Low R:FR light enhances the expression of a positive regulator of the brassinosteroid (BR) signalling pathway, *BRASSINOSTEROID SIGNALLING KINASE 5* (*BSK5*). We found that ABA inhibits *BSK5* up-regulation and interferes with GSK3-like kinase inactivation by the BR pathway, thus leading to a suppression of PIF function. By demonstrating a link between the ABA and BR-signalling pathways this study provides an important step forward in our understanding of how environmental cues are integrated into plant development.

Keywords: Plant Photobiology, Salt Response, Phytohormones, Abscisic acid, Brassinosteroids.

Results and Discussion

Soil salinity is detrimental to plants and often results in a reduction in stem and root biomass accumulation [7]. Most previous studies have, however, focused on the effects of salt at a range of very high concentrations (a median concentration of 150mM NaCl) [8]. We opted for a nuanced approach to investigate how low concentrations of NaCl may affect plant shade-avoidance. Growing plants in tissue culture often masks genes involved in NaCl-sensitivity [9,10], and so plants were grown on soil. *Arabidopsis thaliana* (arabidopsis) seeds were germinated under white light (Wl) for three days, before transfer to new soil that had been pre-treated with NaCl solution. Following this, plants were returned to Wl for a further day to acclimate, before they were shifted to Wl or Wl with supplementary far-red LEDs (+FR). Hypocotyl lengths were measured on day seven (for a schematic diagram of the treatments see Figure S1A). Water or NaCl solution was applied from below and the soil was kept saturated to avoid dehydration.

We observed a strong NaCl-mediated reduction in +FR-induced hypocotyl elongation when plants were watered with as low as 10mM NaCl (Figure 1A). Increasing NaCl concentrations beyond 25mM to 75mM NaCl provided no further inhibition of +FR-induced hypocotyl elongation and so 25 mM NaCl was selected for further investigation. In arabidopsis, NaCl inhibited +FR-induced elongation across 3 days of +FR treatment (Figure S1B). We also found that in adult plants, 25 mM NaCl exerted a strong inhibition of +FR-induced rosette expansion through an inhibition of both petiole and lamina elongation (Figures 1B, 1C, S1C and S1D). Additionally, we found that NaCl inhibited +FR induced elongation in tomato and tobacco seedlings (Figure 1D and 1E), suggesting that this could be a general phenomenon in shade-avoiding plants.

To investigate the molecular basis for NaCl-mediated inhibition of +FR-induced elongation, we studied hypocotyl elongation in arabidopsis seedlings. The induction of hypocotyl elongation by +FR in through *PIF4*, *PIF5* and *PIF7* [3,4,11] (Figure 2A). Single *pif4* or *pif5* appeared similar to the wild type, with strong +FR-induced hypocotyl elongation that was inhibited by NaCl. The *pif4/pif5* double mutant however showed reduced +FR-induced elongation, suggesting that these genes act redundantly (Figure 2A). Notably, the hypocotyl length of the *pif4*/*pif5* mutant in +FR light was the same as NaCl +FR treated wild type plants and NaCl did not further supress elongation in this mutant. PIF7 was the dominant PIF driving +FR-induced hypocotyl elongation in our conditions as the *pif7* single mutant showed no significant +FR-induced elongation and there were no additional effects in the *pif4/pif5/pif7* triple mutant. Taken together these results suggest that NaCl limits +FR-induced hypocotyl elongation mostly through an inhibition of PIF4 and PIF5 function.

Many genes are rapidly upregulated by +FR light. Among them, *PIL1* and *IAA19* are direct PIF4 and PIF5 targets [5,12] and show several conserved G-box elements in their promoters (Figure S2A). We used an existing luciferase reporter line for *PIL1* [13] and generated a new LUC reporter line for *IAA19* to assess the activity of these promoters under +FR and NaCl. Both *PIL1* and *IAA19* reporters were strongly induced by +FR light (Figure 2B and 2C). The *PIL1* promoter was initially induced to the same extent in the presence and absence of NaCl, but by day 2, *PIL1* expression was markedly reduced by NaCl (Figure 2B). The presence of NaCl resulted in reduced +FR-induction of *IAA19* expression from the first exposure to +FR (Figure 2C and Figure S2B).

PIFs promote hypocotyl elongation in +FR light through an increase in auxin signalling [2]. To investigate whether NaCl supressed auxin signalling we generated a *DR5v2:GUS* auxin reporter line. *DR5v2* is a synthetic promoter that was developed as a more sensitive auxin reporter than the widely used *DR5* [14]. We found that *DR5v2:GUS* was indeed auxin responsive, particularly in hypocotyls (Figure S2C and S2D). A two hour +FR treatment induced *DR5v2:GUS* activity in the hypocotyl elongation zone, and this was supressed in plants grown on salt-treated soil. Taken together, these results indicate that NaCl supresses +FR-induced gene expression and increased in auxin activity through an inhibition of PIF4 and PIF5 action.

We next investigated how soil salinity exerts its negative effects on PIF4 and PIF5 signalling. Many of the responses to soil salinity have been shown to be mediated by the hormone ABA [15]. Indeed, we found that in plants lacking four of the ABA receptors (*pyr1/pyl1/pyl2/pyl4*- here referred to as *abaQ* [16]), +FR-induced hypocotyl elongation was not affected by NaCl (Figure 3A, 3B). This was also true for plants lacking the downstream ABA components, Sucrose non-fermenting 1-Related protein Kinase 2.2 (SnRK2.2) and SnRK2.3 [17] (Figure 3C) or the four ABA-responsive element-binding factors (ABFs), here referred to as *arebQ* [18] (Figure 3D). The *arebQ* mutants still showed some NaCl-mediated inhibition of +FR-induced hypocotyl elongation, possibly due to the redundant function of other AREB family members, like ABA INSENSITIVE 5 (ABI5). We found that single *abi5* mutants exhibited elongated hypocotyls in all conditions, but still showed a strong NaCl-mediated inhibition of hypocotyl elongation under +FR light (Figure S3A). These results demonstrate that NaCl-mediated inhibition of +FR-induced hypocotyl elongation relies on an intact ABA signaling pathway.

In addition to these mutant analyses, we found that applying increasing concentrations of ABA directly between the cotyledons had a similar effect as applying increasing NaCl concentrations to the soil (Figure 1A versus S3B). Low concentrations of ABA provided a strong break on +FR-induced hypocotyl elongation, this inhibition was saturated by 10 µM ABA and remained constant until at least 100 µM ABA. As with NaCl, the inhibition of +FR-induced hypocotyl elongation by exogenous ABA was absent in the *abaQ* (Figure 3E) and *snrk2.2*/*snrk2.3* mutants (Figure S3C). Importantly, ABA resulted in no further inhibition of +FR-induced elongation in the *pif4*/*pif5* mutant (Figure 3E), consistent with ABA-effects being mediated by inhibition of PIF4/ PIF5 action. ABA also supressed the induction of *DR5v2:GUS* (Figure S3D) and *PIL1* and *IAA19* promoter activity (Figure 2B and 2C) in response to +FR light. Despite the clear requirement for ABA signalling, NaCl still inhibited +FR-induced elongation in mutants that have reduced levels of ABA synthesis, such as *aba2* and *aba3* (Figure S3E and S3F)and we were unable to detect any increase in ABA levels in NaCl-grown seedlings (Figure S3G).

In the context of seed germination, it is well established that ABA acts antagonistically to the hormone gibberellic acid (GA) [19]. GA promotes cell elongation through the degradation of growth-repressing DELLA proteins. DELLA proteins form competitive heterodimers with PIFs [20,21], and their inactivation is required for +FR-induced elongation to occur [22]. Previous studies have shown that DELLA proteins are stabilised by NaCl [23] and so we investigated whether DELLAs play a role in our experimental system. Mutants that lacked DELLA proteins showed longer hypocotyls than the wild type in every condition tested (Figure S4A), but unexpectedly we found that these plants still exhibit a strong NaCl-mediated inhibition of +FR-induced elongation, suggesting this response is driven at least in part through other mechanisms. Interestingly, more recent studies have found that the stabilisation

of DELLA proteins by NaCl in the root is transient [24] and so it could be that their role in elongation is restricted to growth arrest upon initial NaCl exposure.

Another class of hormones with which ABA displays an antagonistic relationship are the brassinosteroids (BRs) [25] and ABA production has been shown to precede a reduction of BR signalling during NaCl-induced root quiescence [24]. BR signalling is necessary for +FR-induced elongation to occur [11] raising the possibility that ABA and NaCl act by inhibiting BR signals. We tested the effect of brassinazole (BRZ- an inhibitor of BR synthesis) on hypocotyl elongation under +FR light. We found that BRZ, much like both NaCl and ABA inhibited hypocotyl elongation very readily at low concentrations and that this inhibition remained constant as concentrations increased (Figure S4B). Intriguingly, hypocotyl length at this plateau was equal to that of +FR-treated plants grown on saline soil. Furthermore, simultaneous application of ABA, BRZ and NaCl had no further effect on the hypocotyl length of +FR-treated plants than NaCl treatment alone (Figure S4C), consistent with them all acting through the same pathway. Accordingly, application of epi-brassinolide (BL) rescued +FR-induced hypocotyl elongation in NaCl-exposed plants (Figure 4A, S4D). Hypocotyl length was rescued at very low concentrations of epi-BL, and this was to the extent of +FR-treated control plants grown in the absence of NaCl (Figure S4D). Importantly, the rescue of hypocotyl elongation by epi-BL did not occur in the *pif4pif5* mutant, demonstrating that in this context, epi-BL acts through PIF4 and/or PIF5 (Figure 4A).

Crosstalk between the BR pathway and PIFs is well established. PIF4 directly interacts with the BR-activated transcription factor BRASINAZOLE RESISTANT 1 (BZR1), and BZR1:PIF4 heterodimers inter-dependently control the expression of thousands of genes [26]. Additionally, BRASSIONOSTEROID INSENSITIVE 2 (BIN2) (a GSK3-like kinase that acts as a negative regulator of the BR pathway) inactivates BZR1 [27] and also has a direct inhibitory effect on PIF4 [28] and PIF3 [29]. ABA has been shown to enhance the kinase activity of BIN2 against two of its targets, BRI1-EMS SUPRESSOR 1 (BES1- a close relative of BZR1) and ABI5 [30,31].

BIN2 acts redundantly with two other GSK3-like kinases, BIN2-LIKE 1 (BIL1) and (BIL2). We found that a mutant lacking these three proteins (*gsk3 triple*) showed a clearly reduced salt-mediated inhibition of +FR-induced elongation as compared to the wild type (Figure 4B). Additionally, the *gsk3 triple* mutant showed no ABA or BZR-mediated inhibition of +FR-induced elongation, indicating that GSK3 kinases are required in both the ABA and BR signalling pathways in our system (Figure S4F).

Genetic approaches have suggested that ABA enhances BIN2 activity by intercepting the BR signalling pathway at some point between BR perception and the regulation of BIN2 activity [30]. A recent study identified a possible mechanism for ABA-mediated inhibition of BR signalling in rice [32] whereby ABA enhances the expression of the membrane protein *REMORIN 4.1* (*OsREM4.1*) which goes on to block the formation of the BR co-receptor complex. The closest relatives of *OsREM4.1* in arabidopsis are *REM4.1* and *REM4.2* and they too are strongly ABA-upregulated [33]. We found however that mutants lacking both of these genes [34] were indistinguishable from the wild type in our hypocotyl elongation assay (Figure 4E), indicating that they are not required for NaCl-mediated inhibition of +FR-elongation in arabidopsis seedlings.

We therefore turned our attention to BR-signal components between BR perception and the regulation of BIN2. Upon BR perception, a family of receptor-like cytoplasmic kinases, known as

BRASSINOSTEROID SIGNALLING KINASES (BSKs) become activated [35], allowing them to supress GSK3-like kinases [35–37]. Despite the high degree of redundancy between BSKs [37] a recent study found that a mutation in just one of the 12 putative family members, *BSK5*, caused alterations in plant sensitivity to NaCl and ABA [38]. BSK5 may also play a role in shade-avoidance, given that +FR light induces the expression of *BSK5*, particularly in hypocotyls [39]. We found that in our growth conditions, *BSK5* transcripts were indeed upregulated and activation of this gene was redundantly regulated by PIF4, PIF5 and PIF7 (Figure 4C).

The *BSK5* promoter contains a G-box approximately 3.5kb from its start codon (Figure S4G). A previous study has shown that multiple PIFs can bind to this G-Box [12] and a recent large-scale ChIP-seq assay showed that at least one AREB family member, ABF3 binds to the same G-box in an ABA-dependent manner [40]. We therefore investigated whether the expression of *BSK5* was also regulated by ABA. We found that ABA pre-treatment strongly inhibited the +FR-mediated upregulation of *BSK5* transcripts in hypocotyls (Figure 4D). Importantly, ABA repression of *BSK5* transcripts was dependent upon the AREB family of transcription factors.

To test the significance of BSK5 for +FR-induced elongation, we obtained a mutant line deficient in *BSK5* expression [38]. We found that under +FR light in the absence of NaCl, these plants elongated only to the extent of NaCl and +FR treated wild type plants (Figure 4E). The addition of NaCl caused no further inhibition of elongation in these plants, strongly resembling the phenotype of *pif4*/*pif5* mutants (Figure 2A).

Together our results suggest a mechanism whereby +FR light promotes the stabilisation of PIF4, PIF5 and PIF7 (Figure 4F) and these transcription factors then promote the expression of BSK5. The BSK5 kinase supresses the action of GSK3-like kinases, and thereby further promotes the action of PIF4/PIF5. In the presence of soil salinity, activation of the ABA signal transduction pathway results in the promotion of AREB/ ABF transcription factors that suppress BSK5 transcription, and therefore break the +FR-induced PIF/BSK5 feed-forward loop. Lack of the phosphorylation cascade mediated by BSK5, allows activation of GSK3-like kinases and leads to suppression of PIF4/PIF5 function. Future studies should investigate if suppression of PIF4/PIF5 function by GSK3-like kinases occurs via direct phosphorylation or through the suppression of a PIF4 co-factor such as BZR1. There may be an adaptive significance to our finding that plants grown in saline soils supress BR and PIF signalling when they are presented with shade cues. Indeed, a recent study found that overexpression of PIF4 reduces plant survival in saline conditions [41]. It is possible then that shade avoidance signalling is detrimental to salt survival. It is notable that BR-based chemicals are currently being developed as treatments for salt and drought afflicted crops [42]. It may be that under salt and shade conditions, re-activation of the BR signal cascade is detrimental to plant health.

Author Contributions

Conceptualization, S.H, C.T and R.P; Methodology, S.H, A.T, S.P and R.P; Formal analysis, S.H; Investigation, S.H, A.T, K.vG, M.V and S.P; Resources, R.S, S.P and R.P; Writing (original), S.H; Writing (review and editing), S.H, C.T, S.P, R.S and R.P; Visualisation, S.H; Funding acquisition, S.H and R.P.

Materials and Methods

<u>Morphological studies</u>

Plants were sown directly onto wetted soil and stratified for 3-4 days at 4°C in darkness. Plants were moved to growth chambers with a 16:8 hour photoperiod, 130-140 $\mu molm^{-2}s^{-1}$ white light. After 3 days, plants were transferred to new soil that had been previously wetted with deionised water or the indicated concentration of NaCl and then returned to white light for the indicated period. Wl +FR treatments were provided by supplementary FR LEDs to a R:FR ratio of 0.05.

Biological materials

Mutant arabidopsis lines used in this study were as follows: *pif4-101*, *pif5* (*pil6-1*), *pif7-1*, *pif4-101/pif5* (*pil6-1*), *pif4-101/pif5* (*pil6-1*)/ *pif7-1*, *abaQ* (*pyr1-1/pyl1-1/pyl2-1/pyl4-1*), *snrk2.2/snrk2.3*, *arebQ* (*areb1/ areb2/ abf3/ abf1-1*), *bsk5*, *aba2-1*, *aba3-1*, *rem4.1-1*, *rem4.2-1* and *rem4.1-1/ rem2.4-1* in the Columbia ecotype; *abi5-1* and *gsk3-triple* (*bin2-3/bil1-1/bil2-1*) in the Ws ecotype; and *della global* (*gai-t6/ rga-t2/ rgl1-1/ rgl2-1/ rgl3-4*) in the L*er* ecotype. The other species used were tomato (*S.lycopersicum* var. "Moneymaker") and Tobacco (*N. benthamiana*). See table S1.

New lines developed for this paper

pDR5v2:GUS- The *pGREENII0179:pDR5v2:GUS* plasmid was constructed by ligating a *pDR5v2* fragment cut from *pUC57:DR5v2* with EcoRI and BamHI into *pGREENII0179* lacking a promoter. The *GUS* gene was amplified from a *pDR5:GUS* arabidopsis line containing a *GUS* reporter with primers introducing NotI and SacI restriction sites. The PCR fragment was subsequently cut with NotI and SacI and ligated into *pGREENII0179:pDR5v2* to create *pGREENII0179:pDR5v2:GUS*. This was then transformed into *E.coli* (strain- DH5α) which was sequenced to confirm the correct amplification of *GUS*. The *pDR5v2:GUS* arabidopsis line was made transforming *pGREENII0179:pDR5v2:GUS* into *agrobacterium* (strain AGL1) containing the *pSOUP* plasmid. This was then transformed into Col-0 plants by floral dipping, according to an updated protocol [43]. 21 independent transformants were selected by antibiotic resistance and T2 lines screened for single insertions and GUS staining intensity. T3 homozygous lines were selected by kanamycin resistance and checked for signal strength, distribution and auxin responsiveness.

pIAA19:LUC- The *pIAA19:LUC* plasmid was constructed by amplifying the *IAA19* promoter from arabidopsis thaliana (Col ecotype), with the primers listed in the key resources table (see online). The promoter fragment was then cloned into *pENTR_D-TOPO* and mobilized into the LucTrap3 vector. This construct was then transformed into *agrobacterium* (strain AGL1) and used for transformation of Col-0 plants by floral dip.

For oligonucleotides used in this study see table S2.

Gene expression studies

Seedlings were grown in the indicated conditions, before dissection and flash freezing in liquid nitrogen. RNA was extracted with an RNeasy Mini Kit (QIAGEN) with an on-column treatment with DNase (QIAGEN). cDNA was synthesized using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) with random hexamers. qPCR was performed with SYBR green ready mix (Bio-rad) in a ViiA 7 Real-Time PCR System (Thermo Fisher Scientific). Ct values for the gene of interest are expressed relative to that Ct value found for primers targeted to *AT1613320*. For each experiment, 2 technical and a minimum of 3 biological repeats was performed.

For oligonucleotides used in this study see table S2.

Luciferase assay

pPIL1:LUC and *pIAA19:LUC* seedlings were germinated in 1/2 MS media. On cotyledon expansion, seedlings were transferred to microtiter plates containing 170 µl solid ½ MS media (6% agar)supplemented with EtOH (mock), 75 mM NaCl (NaCl), or 1 µM ABA, per well. 30 µl of a Luciferin solution (8 µl of a 10 mg/ml luciferin (Promega) stock in DMSO, diluted in 4 ml H2O) were added per well, and plates were sealed with a sealing film (Applied Biosystems). Two holes were then made with a fine needle per well for seedlings transpiration, and emitted luminescence due to LUC activity

recorded using a Berthold LB960 station installed in a Percival growth chamber. Luminescence was recorded each hour, using 2 seconds read per well, during two consecutive days, and on third day ZT4 light was supplemented with FR.

ABA Extraction and Quantification

20-50 mg seedlings (approximately 50) were grown under the indicated conditions, harvested and flash frozen in liquid nitrogen. ABA was extracted as described in [44] and quantified by liquid chromatography-mass spectrometry (LC-MS) on a Varian 320 Triple Quad LC-MS/MS. ABA levels were quantified from the peak area of each sample compared with the internal standard and normalized by fresh weight.

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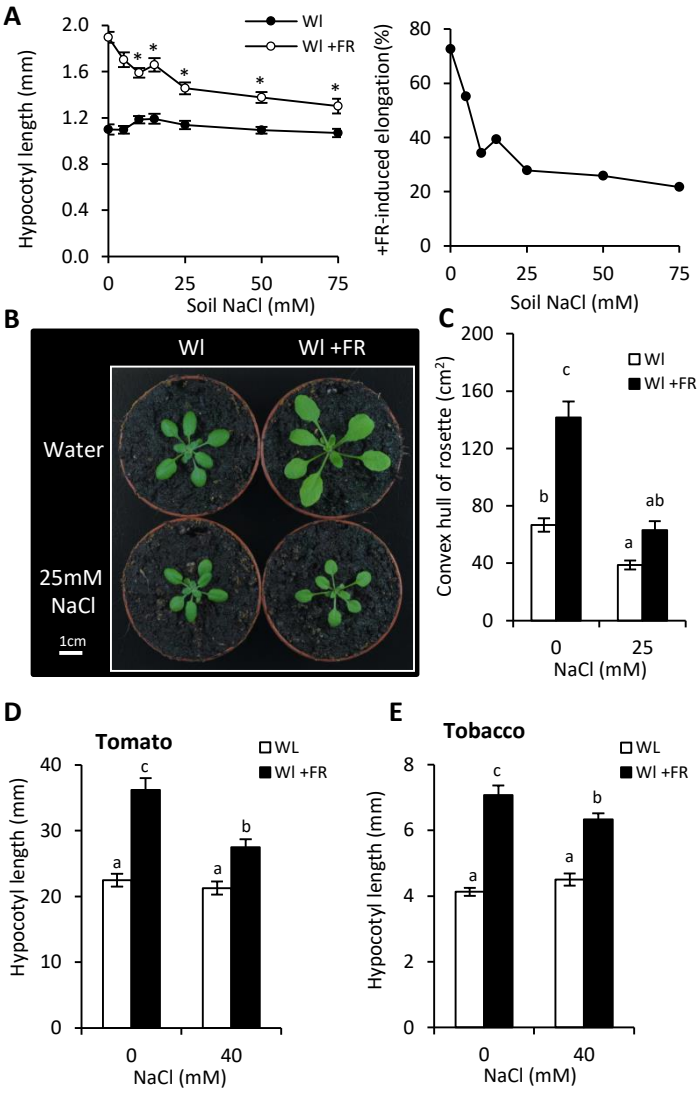


Figure 1. Soil salinity inhibits +FR induced elongation.

(A) Left: Hypocotyl length of 7-day old Col seedlings germinated in Wl, transferred to NaCl soil of the indicated concentration at day 3 and then shifted to WL ±FR at day 4. Data represent mean (n≥19) ± SE. Asterisks indicate salt-mediated inhibition of hypocotyl elongation compared to 0mM NaCl control -Students t-test ($p < 0.05$). Right: The same data expressed as the % +FR-induced elongation at each salt concentration.

(B) Representative 21-day old Col plants germinated in Wl and transferred to ±25mM NaCl at day 3, before shifting to WL ±FR at day 10.

(C) Rosette circumference of plants grown as in B. Data represent mean (n≥10) ± SE. Different letters designate significantly different means by 1-way ANOVA + Tukey's post-hoc test ($p < 0.05$).

(D) Hypocotyl length of 11 day old tomato (*Solanum lycopersicum* var. "Moneymaker") seedlings germinated in Wl, transferred to ±40mM NaCl at day 7, and shifting into WL ±FR at day 8. Data represent mean (n≥19) ± SE

(E) Hypocotyl length of tobacco (*Nicotiana benthamiana*) seedlings grown as in D. Data represent mean (n≥18) ± SE.

Different letters designate significantly different means by 1-way ANOVA + Tukey's post-hoc test ($p < 0.05$).

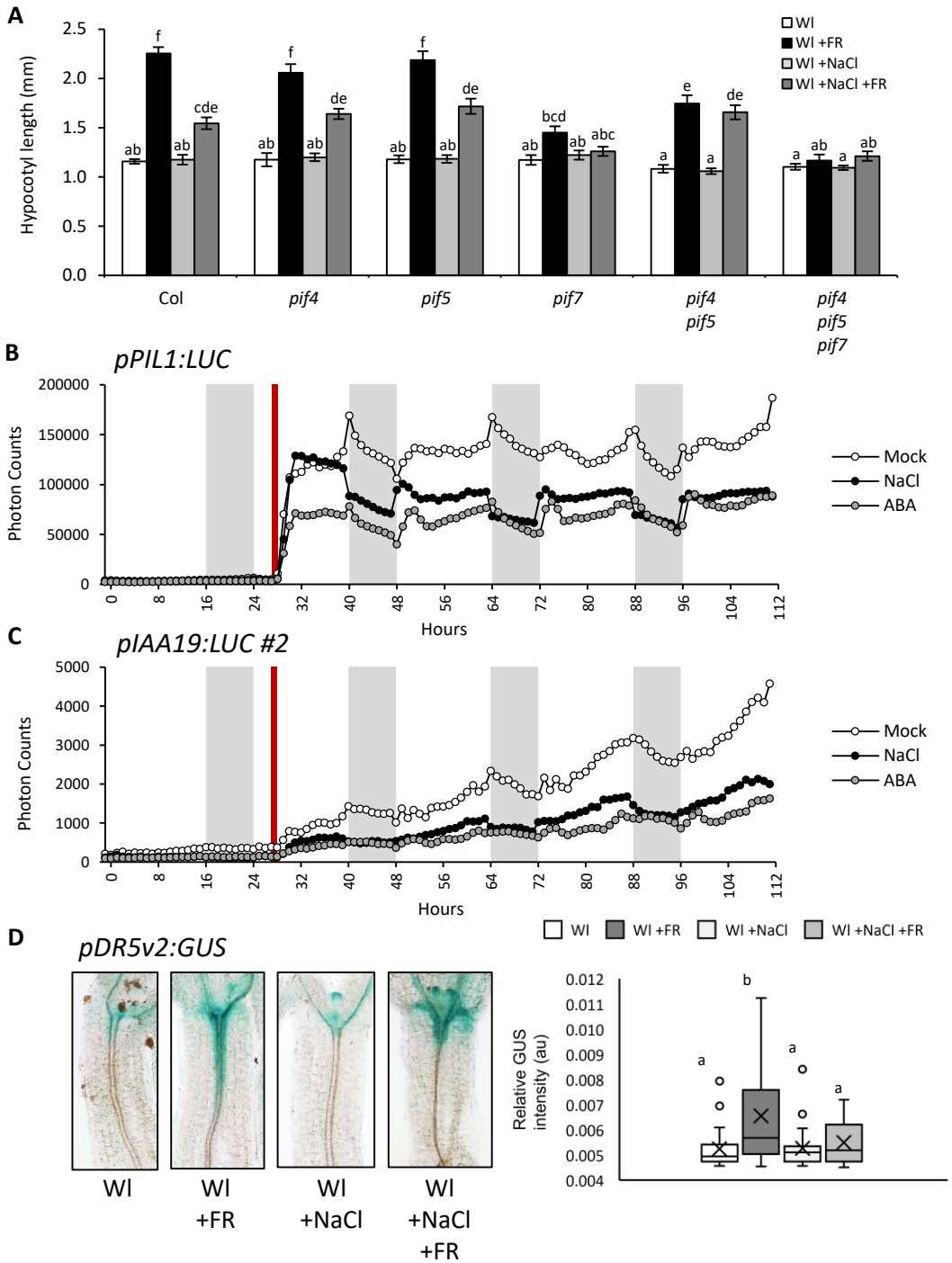


Figure 2. Soil salinity supresses PIF-function and +FR-induced gene expression and auxin signalling

(A) Hypocotyl length of 7-day old Col, *pif4-101*, *pif5* and *pif7-1* double and triple mutant seedlings germinated in Wl, transferred to 25mM NaCl soil at day 3 and then shifted to WL ±FR at day 4. Data represent mean (n≥23) ± SE. Different letters designate significantly different means by 1-way ANOVA + Tukey's post-hoc test ($p < 0.05$).

(B) Seedlings expressing a *pPIL1:LUC* reporter were germinated on half MS media for 3 days, before transfer to a 96 well plate containing half MS supplemented with luciferin, 75mM NaCl, 2 µM ABA and/or ethanol control. Luciferase activity was recorded for two days (one day shown for clarity) before supplementary FR LEDs were switched on (red line) 4 hours after dawn on day 3. Luciferase activity was then recorded for further 3 days. Grey bars represent night (16:8 h photoperiod).

(C) Luciferase activity of seedlings expressing a *pIAA19:LUC* reporter (line #2), grown as in B.

(D) Left: Representative GUS-stains of seedlings expressing a *pDR5v2:GUS* reporter. Seedlings were germinated on soil in Wl for 3 days before being transferred to soil treated with 25mM NaCl for a further day. At 2.5 hours after dawn (ZT 2.5) on day 4, plants were shifted into Wl +FR or control light cabinets and samples were harvested at ZT 4.5. Right: Quantification of GUS staining from images of >23 plants. X represents the mean, horizontal bars, boxes, and whiskers show medians, interquartile ranges and data ranges, respectively. Circles mark outliers. Different letters designate significantly different means by 1-way ANOVA + Tukey's post-hoc test ($p < 0.05$).

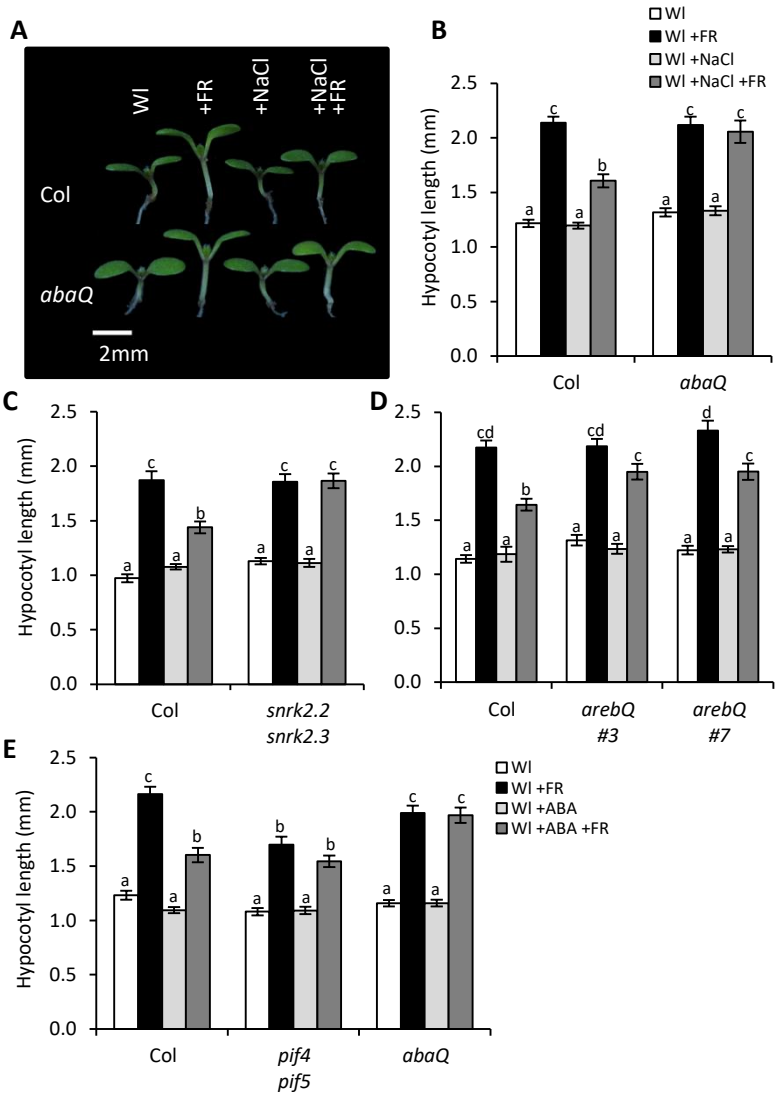


Figure 3. Salinity-mediated inhibition of +FR-induced elongation requires ABA signalling

(A) Representative samples of 7-day old Col and *abaQ* seedlings germinated in WI, transferred to ±25mM NaCl soil at day 3 and then shifted to WL ±FR at day 4.

(B) Hypocotyl length seedlings grown in A. Data represent mean (n=22) ± SE.

(C) Hypocotyl length of Col and *snrk2.2/snrk2.3* double mutant grown as in A. Data represent mean (n ≥ 23) ± SE.

(D) Hypocotyl length of Col and two independent *arebQ* lines grown as in A. Data represent mean (n ≥ 23) ± SE.

(E) Hypocotyl length of Col, *pif4-101/pif5* and *abaQ* seedlings grown 3 d in WI, transferred to new pots for 1 d WI, then treated 3 d WL ±FR. 1 µl 25 µM ABA applied to each seedling on d 3-6. Data represent mean (n = 24) ± SE. Different letters designate significantly different means by 1-way ANOVA + Tukey's post-hoc test ($p < 0.05$).

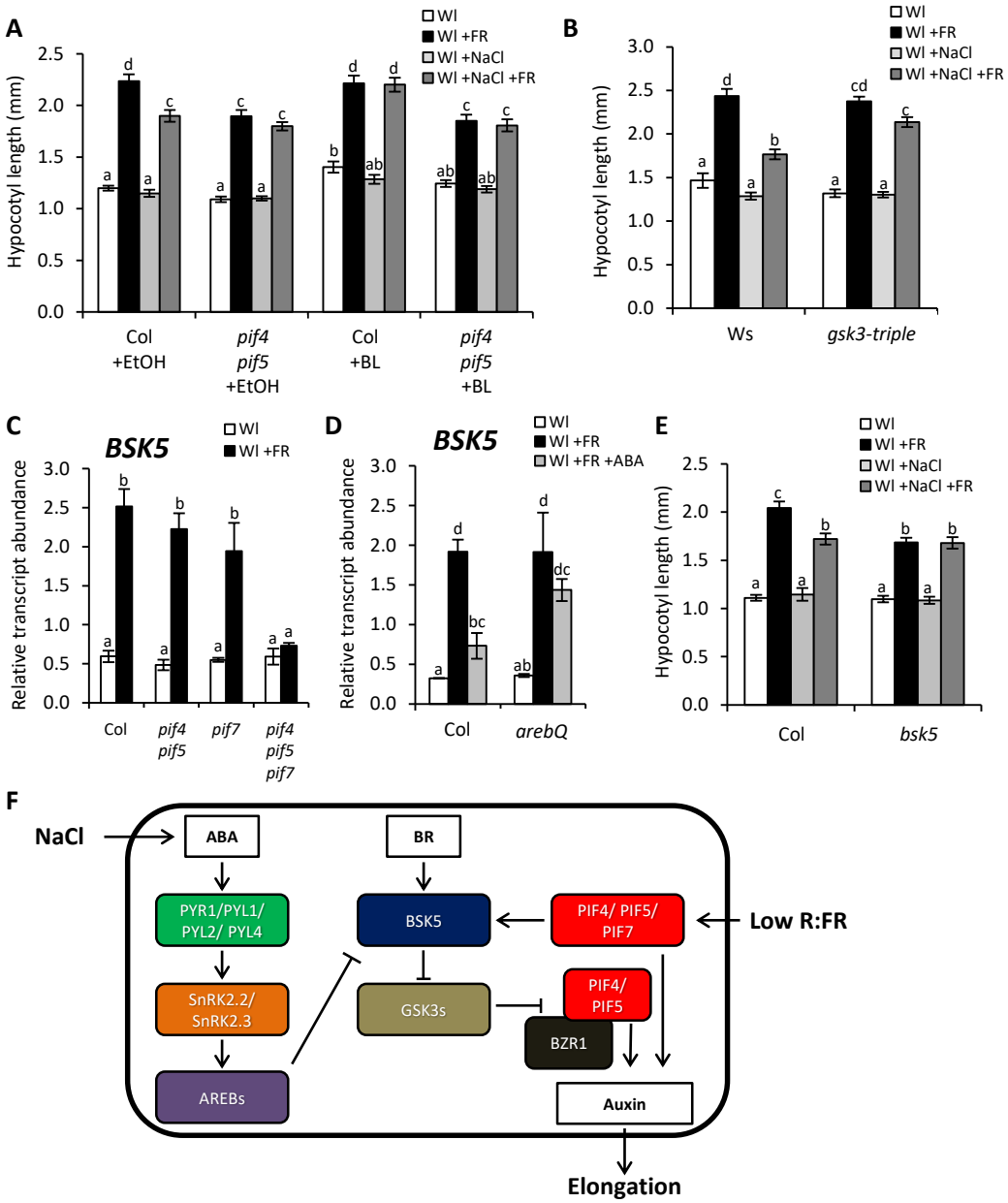


Figure 4. ABA and NaCl inhibit +FR elongation through inhibiting BR signalling

(A) Hypocotyl length of 7-day old Col and *pif4-101/pif5* seedlings germinated in WI, transferred to ±25mM NaCl soil at day 3 and then shifted to WL ±FR at day 4. Plants were sprayed with 10 µM epi-BL or ethanol control on days 3-6. Data represent mean (n ≥ 46) ± SE.

(B) Hypocotyl length of Ws and *gsk3-triple* mutant grown as in A, without hormone applications. Data represent mean (n ≥ 24) ± SE.

(C) Col and *pif4-101/pif5*, *pif7-1* and *pif4-101/pif5/pif7-1* plants were germinated in WI for 3 days and then transferred to new pots. At ZT 2.5 on day 7, plants were shifted to WI ±FR light cabinets. At ZT 4.5, the hypocotyls of approximately 20 seedlings per sample were dissected and RNA extracted. *BSK5* transcripts relative to *AT1613320* are shown. Data represent mean (n=3) ± SE.

(D) Col and *arebQ #3* plants were grown for germinated in WI for 3 days and then transferred to new pots. At ZT 1.5 on day 7, plants were sprayed with 25 µM ABA. At ZT 2.5 plants were exposed to WI ±FR. At ZT 4.5, the hypocotyls of approximately 20 seedlings per sample were dissected and RNA extracted. *BSK5* transcripts relative to *AT1613320* are shown. Data represent mean (n=3) ± SE.

(E) Hypocotyl length of Col and *bsk5* grown as in B. Data represent mean (n ≥ 22) ± SE.

Different letters designate significantly different means by 1-way ANOVA + Tukey's post-hoc test ($p < 0.05$).

(F) A proposed mechanism for salt-mediated inhibition of low R:FR-induced elongation. Low R:FR light promotes the stability of PIF4, 5 and 7. These PIFs redundantly upregulate the expression of *BSK5*. BSK5 acts to supress the activity of GSK3-like kinases, which relieves their suppression of the PIF:BZR1/BES1 signalling module in a positive feedback loop. NaCl promotes the canonical ABA signal transduction pathway, resulting in the increased activity of AREB/ABF transcription factors. AREB/ABFs inhibit the upregulation of *BSK5* and thereby promote GSK3-like kinase action. GSK3-like kinases are then able to inhibit PIF4/PIF5 function posibly through direct phosphorylation/ destabilisation or through inhibition of their co-activator BZR1, resulting in reduced auxin activity and a suppression of hypocotyl elongation.

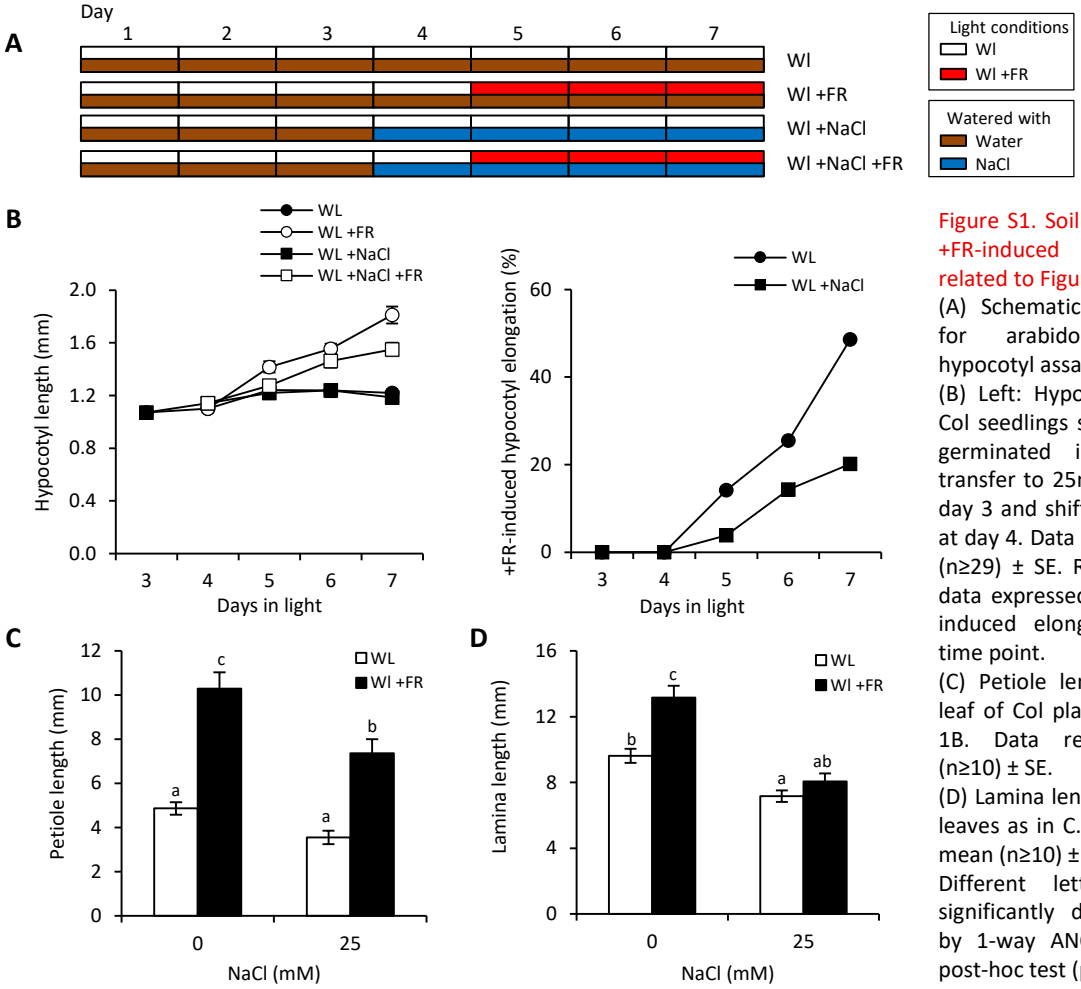


Figure S1. Soil salinity inhibits +FR-induced elongation; related to Figure 1

(A) Schematic of treatments for arabidopsis seedling hypocotyl assays

(B) Left: Hypocotyl length of Col seedlings seedlings grown germinated in Wl, before transfer to 25mM NaCl soil at day 3 and shifted into Wl +FR at day 4. Data represent mean (n≥29) ± SE. Right: The same data expressed as the % +FR-induced elongation at each time point.

(C) Petiole length of the 5th leaf of Col plants as in Figure 1B. Data represent mean (n≥10) ± SE.

(D) Lamina length of the same leaves as in C. Data represent mean (n≥10) ± SE.

Different letters designate significantly different means by 1-way ANOVA + Tukey's post-hoc test (p <0.05).

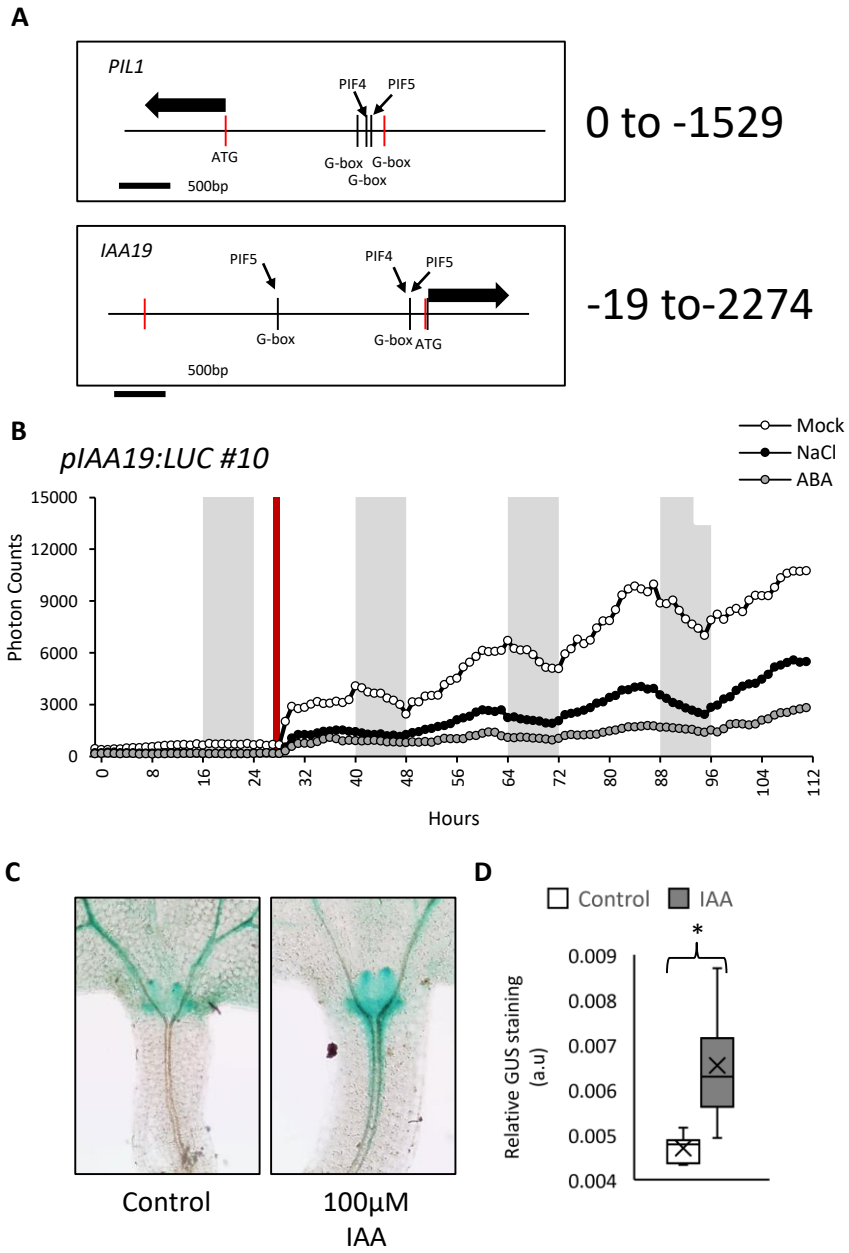


Figure S2. *Additional information on luciferase and GUS reporter lines; related to Figure 2.*

(A) Schematic diagram of the genomic DNA used for the *pPIL1:LUC* and *pIAA19:LUC* reporters. Promoter region cloned is represented between the red lines. ATG site, G-boxes and known PIF binding sites are highlighted.

(B) Independent transgenic line expressing a *pIAA19:LUC* reporter (line #10) were germinated on half MS media for 3 days, before transfer to a 96 well plate containing half MS supplemented with luciferin, 75mM NaCl, 2 µM ABA and/or ethanol control. Luciferase activity was recorded for two days (one day shown for clarity) before supplementary FR LEDs were switched on (red line) 4 hours after dawn on day 3. Luciferase activity was then recorded for further 3 days. Grey bars represent night (16:8 h photoperiod).

(C) Representative GUS stains of plants expressing pDR5v2:GUS treated with auxin. Seeds were germinated in WI on soil for 3 days before being transferred to new soil and returned to WI. On day 4 at ZT 2.5, plants were sprayed with 100µM IAA or an ethanol control and samples we fixed at ZT 4.5.

(D) Quantification of GUS intensity from images of plants treated as in C (n=11). Asterisks denote a significant difference between samples (Students t-test- $p<0.001$).

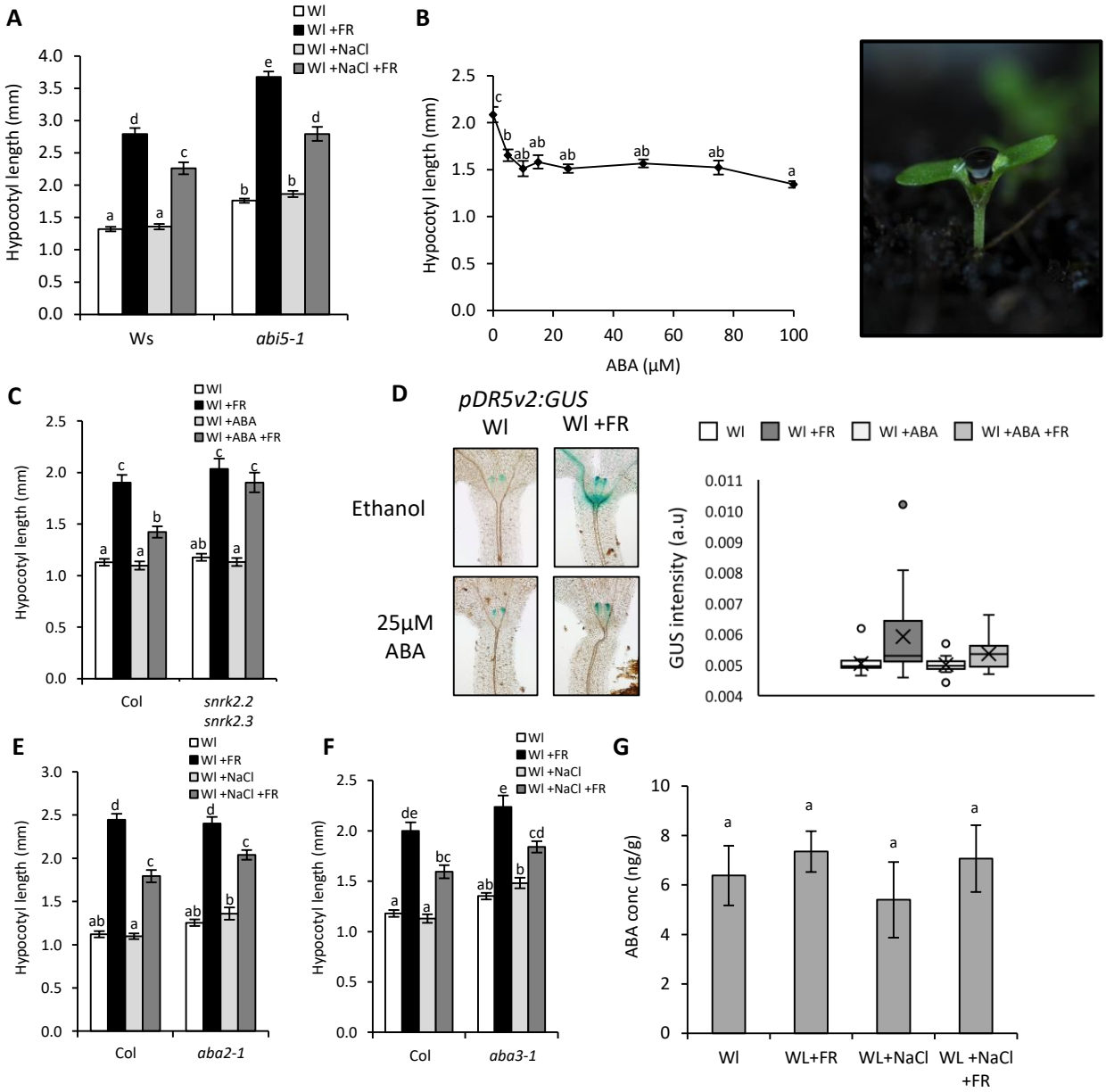


Figure S3. Salt inhibits +FR-induced hypocotyl elongation through ABA signalling, but this can still occur in several ABA mutants; related to Figure 3

(A) Hypocotyl length of 7-day old Ws and *abi5-1* seedlings germinated in Wl, transferred to ±25mM NaCl soil at day 3 and then shifted to WL ±FR at day 4. Data represent mean (n =30) ± SE.

(B) Left: Hypocotyl length of 7-day old Col seedlings germinated in Wl, transferred to new soil at day 3 and then shifted to WL +FR at day 4. 1 µl of the indicated concentration ABA was applied between the cotyledons on days 3-6. Data represent mean (n=24) ± SE. Right: Representative image of ABA treatments.

(C) Hypocotyl length of 7-day old Col and *snrk2.2/snrk2.3* seedlings germinated in Wl, transferred to new soil at day 3 and then shifted to WL ±FR at day 4. 1 µl 25 µM ABA applied to each seedling on d 3-6. Data represent mean (n = 24) ± SE.

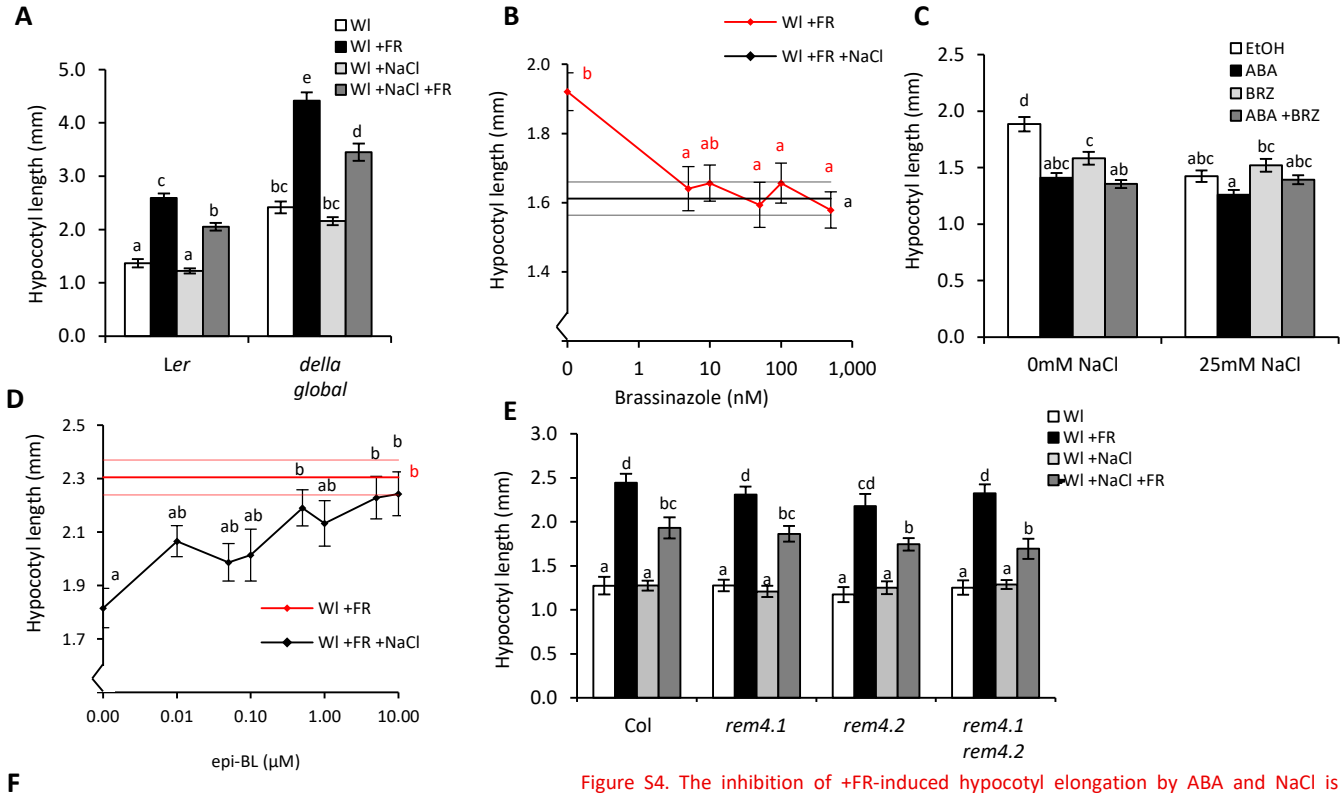
(D) Left: Representative GUS-stains of seedlings expressing a *pDR5v2:GUS* reporter. Seedlings were germinated on soil in Wl for 3 days before being transferred to new soil for a further day in Wl. At ZT 1.5 on day 4, plants were sprayed with 25µM ABA or an ethanol control. At ZT 2.5, plants were shifted into Wl +FR or control light cabinets and samples were harvested at ZT4.5. Right: Quantification of GUS staining from images of >16 plants. X represents the mean, horizontal bars, boxes, and whiskers show medians, interquartile ranges and data ranges, respectively. Circles mark outliers.

(E)Hypocotyl length of Col -0 and *aba2-1* seedlings grown as in A. Data represent mean (n =30) ± SE.

(F) Hypocotyl length of Col -0 and *aba3-1* seedlings grown as in A. Data represent mean (n ≥23) ± SE.

(G) ABA content of 7-day old Col plants germinated in Wl, transferred to ±25mM NaCl soil at day 3 and then shifted to WL ±FR at day 4. the stems (aerial portion) of approximately 50 seedlings were harvested on day 7, 4 hours post dawn. Data represent mean (n=6) ± SE.

Different letters designate significantly different means by 1-way ANOVA + Tukey's post-hoc test ($p < 0.05$).



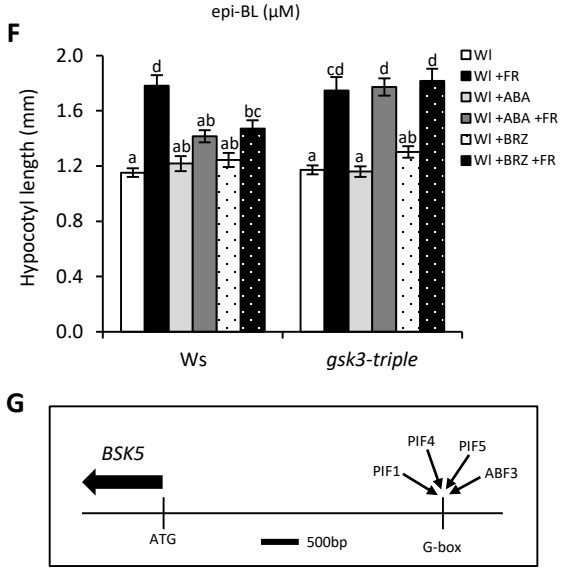


Figure S4. The inhibition of +FR-induced hypocotyl elongation by ABA and NaCl is mediated through a reduction in BR signalling; related to Figure 4.

(A) Hypocotyl length of 7-day old L*er* and *della global* (pentuple) mutant germinated in Wl, transferred to ±25mM NaCl soil at day 3 and then shifted to WL ±FR at day 4. Data represent mean (n ≥17) ± SE.

(B) Hypocotyl length of 7-day old Col seedlings germinated in Wl, transferred to ±25mM NaCl soil at day 3 and then shifted to WL +FR at day 4. 1 µl of the indicated concentration brassinazole was applied between the cotyledons on days 3-6. Data represent mean (n≥52) ± SE. The black horizontal lines indicate the mean hypocotyl length (± SE) of control plants transferred to 25mM NaCl soil and treated with mock applications; demonstrating that brassinazole inhibits hypocotyl elongation to the same extent as salt.

(C) Hypocotyl length of 7-day old Col seedlings germinated in Wl, transferred to ±25mM NaCl soil at day 3 and then shifted to WL +FR at day 4. 1 µl ethanol control, 25 µM ABA, 50nM brassinazole (BRZ) or a combination of both was applied to each seedling on d 3-6. Data represent mean (n > 32) ± SE.

(D) Hypocotyl length of 7-day old Col seedlings germinated in Wl, transferred to ±25mM NaCl soil at day 3 and then shifted to WL +FR at day 4. The indicated concentration epi-brassinolide was sprayed onto the plants on days 3-6. Data represent mean (n≥32) ± SE. The red horizontal lines indicate the mean hypocotyl length (± SE) of control plants transferred to non-NaCl soil and treated with mock applications; demonstrating that epi-brassinolide promotes hypocotyl elongation same extent as in non-salt treated plants.

(E) Hypocotyl length of Col and *rem4.1* and *rem4.2* single and double mutants grown as in A. Data represent mean (n ≥21) ± SE.

(F) Hypocotyl length of 7-day old Ws and *gsk3-triple* seedlings germinated in Wl, transferred to ±25mM NaCl soil at day 3 and then shifted to WL ±FR at day 4. 1 µl 25 µM ABA and/or 50nM BRZ was applied to each seedling on d 3-6. Data represent mean (n ≥ 25) ± SE.

(G) A schematic diagram of the BSK5 promoter, demonstrating G-boxes and PIF and ABF binding peaks previously identified in reference 12 and 40, respectively.

Different letters designate significantly different means by 1-way ANOVA + Tukey's post-hoc test (p <0.05).