The transcription factor AtHB23 modulates starch turnover for root development and plant survival under salinity

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Highlight: The transcription factor AtHB23 is crucial for plant survival and adaptation to salt stress conditions, and its function is related to the gravitropic response mediated by starch-granule turnover, involving LAX3.

1 **Abstract**

2 AtHB23 is a homeodomain-leucine zipper I transcription factor, previously
 3 characterized as a modulator of lateral root initiation and higher-order roots
 4 development. The role of this gene in response to salinity stress was completely
 5 unknown. To elucidate the role of AtHB23 in response to salinity stress, we combined
 6 histochemical β -glucuronidase (GUS) analysis, root phenotyping, starch staining, optic
 7 and electronic transmission microscopy, expression studies by RT-qPCR, and
 8 transcriptome analysis of silenced, overexpressor, and crossed plants. We revealed
 9 that the expression pattern of *AtHB23* is regulated by NaCl in the main and lateral
 10 roots, affecting the root phenotype. A severe reduction in primary root length, a
 11 significant increment in the initiation of lateral roots, and a low survival rate in salinity
 12 conditions were observed in *AtHB23*-silenced plants, whereas *AtHB23* overexpressors
 13 showed the opposite phenotype. These developmental defects were explained by the
 14 degradation of starch granules and an alteration in starch metabolism. The AtHB23-
 15 target gene *LAX3* is repressed in the tip of the main root and affected by NaCl.
 16 We conclude that AtHB23 is vital for plant survival and adaptation to salt stress
 17 conditions, and its function is related to the gravitropic response mediated by starch
 18 granule turnover, involving the auxin carrier LAX3.

Key words: AtHB23; homeodomain-leucine zipper; HD-Zip I; LAX3; root development;
 root amyloplasts; salinity stress; starch turnover

Abbreviations: HD-Zip, homeodomain-leucine zipper; TF, transcription factor; COL 0,
 wild type; GUS, β -glucuronidase

19 Introduction

20 As sessile organisms, plants must adapt to the soil, climate, and light conditions, as
 21 well as to other biotic and abiotic environmental factors in each particular location.
 22 Among stressor factors, salinity is one of the major causes of poor crop yields. The
 23 situation is further aggravated by undeveloped irrigation strategies, rising population,
 24 and pollution from industrial activity. However, plants evolved the capacity to adapt to
 25 the soil conditions, displaying various physiological and molecular strategies ultimately
 26 integrating external and endogenous signals. Roots are the anchorage organs able to
 27 alter their development, extending through the soil to optimize water and nutrient
 28 uptake (de Dorlodot *et al.*, 2007). Their high plasticity, governed by physiological,
 29 genetic, and epigenetic programs, enables the adaptation to changing environments.
 30 The integration of various cues allows them to balance growth, development, and
 31 stress responses (Schachtman and Goodger, 2008). In this interplay of external and
 32 internal signals, different biomolecules, such as hormones and transcription factors
 33 (TFs), play crucial roles.

34 Although phytohormones have been classified as stress or growth-promotion related
 35 (Verma *et al.*, 2016), numerous studies showed that hormones may participate in
 36 alternative pathways and responses, depending on the developmental stage, or
 37 environmental condition (Yu *et al.*, 2020). Absciscic acid (ABA) is considered the main
 38 abiotic stress hormone and has been shown as a crucial player in the responses to
 39 drought, salinity, and extreme temperatures, among other factors. Studies about the
 40 ABA signaling cascade indicated that this hormone promotes stomata closure which
 41 has a high impact on drought response and photosynthesis rate (Vishwakarma *et al.*,
 42 2017).

43 Under salinity stress, in roots, ABA participates in an intricate crosstalk, notably
 44 involving auxin. It was suggested that the inhibition of root growth by NaCl is
 45 associated with a reduction in auxin content in the tip, mainly due to the alteration of
 46 the auxin carriers AUX1 and PIN2 (Liu *et al.*, 2015). The development of lateral roots
 47 (LR) is also affected by salinity, exhibiting an enhanced growth under low NaCl
 48 concentration (≤ 50 mM) and repressed at higher concentrations. Notably, these
 49 effects are dependent on auxin transport and distribution (Zolla *et al.*, 2010).

50 In early root developmental stages, auxin is transported toward the tip through the
51 central cylinder (Friml *et al.*, 2003) by specific carriers (AUX1, LAX1, LAX2, and LAX3),
52 which exhibit differential expression patterns (Péret *et al.*, 2012; Swarup and Péret,
53 2012). Among these carriers, AUX1 has been linked to LR initiation and LAX3 to LR
54 emergence (Marchant *et al.*, 1999, 2002). Efflux/influx auxin carriers are also involved
55 in root gravitropism, which depends on a differential hormone gradient between the
56 upper and lower sides of the root (Zhang *et al.*, 2019). This gradient regulates the
57 synthesis of starch granules, a process severely affected by salinity (Korver *et al.*, 2020;
58 Zhang *et al.*, 2019). In this way, the gravitational potential energy is converted into a
59 biochemical signal involving statoliths, which are compact starch aggregates formed in
60 the columella cells (Leitz *et al.*, 2009). Besides the gravitropic response, roots also
61 display hydrotropism, interfering with gravitropism in a complex and unknown way.
62 During the hydrotropic response, amyloplasts are degraded in the columella cells
63 reducing the gravitropic response (Nakayama *et al.*, 2012).

64 The complex molecular events modulating root architecture and plasticity involve TFs
65 playing different roles in the adaptation to the environment. TFs families involved in
66 these processes include ARF (Auxin Response Factors), LBD (Lateral Organ Boundaries
67 (LOB) Domain), WRKY (named after the four common WRKY amino acids and a zinc
68 finger-like motif), MYB (MYeloBlastosis oncogene), HD (Homeodomain), bHLH (Basic
69 Helix–Loop–Helix), NAC (NAM, ATAF, and CUC), AP2/ERF Domain (APetala 2/Ethylene-
70 Responsive element-binding Factor), MADS (named after MCM1; AGAMOUS;
71 DEFICIENS, and SRF members), GRF (Growth-Regulating Factor), and GRAS (named
72 after the first three members GIBBERELLIC-ACID INSENSITIVE (GAI), REPRESSOR of GAI
73 (RGA) and SCARECROW (SCR) members (González, 2016; Hong, 2016), which have
74 been functionally characterized in various plant species (T Li *et al.*, 2020; Renau-
75 Morata *et al.*, 2020). Notably, members from these families are interconnected
76 through protein-protein or protein-DNA interactions to activate full transcriptional
77 programs. In particular, members of the ARF and LBD families have been identified as
78 master regulators of LR development, and several among them also mediate the
79 crosstalk between hormones (Friml *et al.*, 2003; Xu *et al.*, 2020; Banda *et al.*, 2020;
80 Lavenus *et al.*, 2013; Lee *et al.*, 2009).

81 The homeodomain-leucine zipper family (HD-Zip) is unique to the plant kingdom. The
 82 members of this family have been classified into four subfamilies according to
 83 structural and functional features (Capella *et al.*, 2015). Many of them participate in
 84 root development (reviewed by Perotti *et al.*, 2021). Particularly, HD-Zip I TFs were
 85 associated with abiotic stress responses, although most studies have been focused on
 86 the aerial organs of the plant (Perotti *et al.*, 2021).

87 AtHB23 is a transcription factor belonging to the HD-Zip I subfamily, and it was
 88 described as a modulator of LR initiation through the direct regulation of *LAX3* and
 89 *LBD16*. This gene is regulated by ARF7/19 (Perotti *et al.*, 2019, 2020). According to
 90 publicly available databases, the expression of *ARF19* is affected by salinity
 91 (<http://bar.utoronto.ca/>; Dinnery *et al.*, 2008), and because HD-Zip I TFs were
 92 associated with abiotic stress (Perotti *et al.*, 2017), we wondered whether AtHB23
 93 participates somehow in the response to salinity in Arabidopsis roots.

94 In this work, we show that *AtHB23* expression is regulated by NaCl, both in primary
 95 and LRs. *AtHB23*-silenced (*amiR23*) plants exhibit an enhanced sensitivity to NaCl
 96 treatments affecting the gravitropic response and survival. Accordingly, starch granules
 97 were degraded when plants were treated with NaCl and were unable to recover once
 98 transferred back to normal conditions. Transcriptomic analyses revealed that AtHB23-
 99 dependent reprogramming in response to salt includes the regulation of starch
 100 turnover related genes. Moreover, we demonstrate that the AtHB23 target gene *LAX3*
 101 is also regulated by salinity and participates in the root adaptive response.

102 **Materials and methods**

103 Genetic constructs

104 *35S::amiR23*, *35S::AtH23*, *prAtHB23:GUS*, *prLBD16:GUS*, *prLAX3:GUS*, and *35S::amiR23*
105 *x prLAX3:GUS* were previously described (Ribone *et al.*, 2015; Perotti *et al.*, 2019).
106 *prAtHB23Δ1273:GUS:GFP*: a PCR reaction using specific oligonucleotides (Table S1) and
107 genomic DNA was carried out. The amplicon (1273 bp upstream the ATG) was cloned
108 into the *pENTR3C* plasmid using the *Bam*H1 and *Eco*RI restriction sites. After that, a
109 Gateway® (Invitrogen) recombination was performed in the destination
110 *pKGWFS7*vector. The resulting construct was called *prAtHB23_s:GUS*.

111

112 Plant material, growth conditions, and transformation

113 For NaCl treatments and root phenotyping, *Arabidopsis thaliana* plants (Col 0 ecotype)
114 were grown in a growth chamber at 22–24°C under long-day conditions (16/8 h
115 light/dark cycles) with a light intensity of approximately 70 μmol m⁻² sec⁻¹ in vertical
116 square Petri dishes (12 x 12 cm) with Murashige–Skoog medium supplemented with
117 vitamins (MS; PhytoTechnology Laboratories, <https://phytotechlab.com/home>).

118

119 Histochemical GUS staining

120 Plants were immersed in GUS staining buffer (1 mM 5-bromo-4-chloro-3-indolyl-GlcA
121 in 100 mM sodium phosphate, pH 7, 0.1% (v/v) Triton X-100, and 100 mM potassium
122 ferrocyanide), vacuum was applied for 5 min, and then plants were incubated at 37°C
123 for 12 h. Chlorophyll was cleared from the plant tissues by immersion in 70% ethanol.

124

125 Root phenotyping

126 Seeds were surface sterilized and placed 1 cm from the top for 3 days at 4°C before
127 placing the dishes in the growth chamber. The lengths of the main root were measured
128 using the RootNav free software from photographs of the plates (Pound *et al.*, 2013).

129

130 Salinity treatments

131 Assays done with *prAtHB23:GUS* plants: seedlings were grown in normal conditions in
132 vertical Petri dishes (12 x 12 cm) and then placed in plates with the same medium
133 supplemented with NaCl (concentrations indicated in each Figure legend). LR and LRP

134 observation and counting were done with seedlings grown in normal conditions during
135 3 days and placed in plates supplemented with NaCl for additional 5 days.

136 The survival experiment was performed with plants placed in 100 mM NaCl after three
137 days of growing in normal conditions.

138 To amyloplast observation and transcript quantification, 5-day-old plants grown in
139 control conditions were treated during 8 h with 150 mM NaCl. After that, half of the
140 plants were transferred to control conditions and the other half remained in the
141 salinity medium during 72 additional hours.

142

143 Amyloplasts Staining and Light Microscopy Observation

144 To observe the amyloplasts in the columella cells of the root tips, 15-20 Arabidopsis
145 roots (5 day-old) were dipped in Lugol staining solution (Sigma-Aldrich) for 8-10 min,
146 and then observed in an Eclipse E200 Microscope (Nikon, Tokyo, Japan,
147 <https://www.nikon.com/>) equipped with a Nikon Coolpix L810 camera.

148

149 RNA isolation and analysis

150 The total RNA used in quantitative reverse transcriptase RT-qPCR was isolated from
151 Arabidopsis roots using Trizol reagent (Invitrogen,
152 <https://www.thermofisher.com/ar/>), according to the manufacturer's instructions.

153 One microgram of RNA was reverse transcribed using oligo(dT)₁₈ and M-MLV reverse
154 transcriptase II (Promega, <https://worldwide.promega.com/>). Quantitative real time
155 PCR (qPCR) was performed using a StepOnePlus Real-Time Systems of Applied
156 Biosystems TM. Each reaction contained 10 µl final volume having 5 µl Taq TM SYBR®
157 Green Supermix, 0,2 µl of each specific oligonucleotide (10 pmol/µl; Table S1) and 1/20
158 of RT product. Fluorescence was continuously recorded at 72°C during 40 cycles. The
159 quantification of mRNA levels was achieved by normalization against ACTIN transcripts
160 levels (*ACTIN2* and *ACTIN8*), following the $\Delta\Delta C_t$ method. All reactions were performed
161 with at least three biological replicates and bars represent SEM.

162

163 Histology and electronic microscopy

164 The roots were fixed in glutaraldehyde at 2% (v / v) in 0.2 mM potassium phosphate
165 buffer (pH 7.4) and kept at room temperature for 2 h (under vacuum) and then

brought to 4°C overnight. Segment were rinsed with the same buffer and subsequently fixed in 2% (w / v) OsO₄ in buffer (pH 7.0) at room temperature for 2 h (under vacuum), and then dehydrated in a series of acetone and embedded in Durcupan resin (whit vacuum application at each step). For light microscopy, sections about 0.5 µm thick were stained with 0.05% (w / v) toluidine blue in bidistilled water. For transmission electron microscopy, ultrathin sections (70-90 nm in thickness) were stained with uranyl acetate and lead citrate and observed under a Philips EM201 transmission electron microscope (TEM) at an acceleration voltage of 60-80 kV.

174

175 Transcriptome analysis

176 For transcriptome analyses, Col 0 and *amiR23* seeds were grown for 3 days in normal conditions. After that one half of the plates were transferred to new MS plates supplemented with 75 mM NaCl and samples were harvested 6 days after that. Total RNA was isolated from roots. The experiment was performed with biological replicates of each genotype/treatment. Isolated RNA was lyophilized before shipping.

181 Sequencing was performed by the BGI Genomics Sequencing Service (https://www.bgi.com/global/), using the DNBseq platform, and obtaining 100 nucleotide long paired-end reads. Raw reads were first quality trimmed and filtered with Trimmomatic (version 0.36; Bolger *et al.*, 2014) and then aligned to the *Arabidopsis thaliana* genome (TAIR10) using STAR (version 2.5.2b, Dobin *et al.*, 2014), which was guided by the gene and exon annotation from Araport (V11 201606, Pasha *et al.*, 2020). Samtools (version 1.8; Li *et al.*, 2009) was then used to keep only primary alignments with a minimum MAPQ of 3. Read quality before and after trimming was analyzed with FastQC (version 0.11.5; Andrews 2010) and, together with mapping efficiency, it was summarized with MultiQC (version 1.7; Ewels *et al.*, 2016). Read counts on each gene were calculated with featureCounts (version 1.6.2; Liao *et al.*, 2014). This pipeline was run with the aid of the Snakemake workflow engine (Köster and Rahmann, 2012). Differentially expressed genes were determined with DESeq2 (Love *et al.*, 2014; R Core Team, 2020) filtering out genes with counts below 10 in all samples, and combining factors (i.e. genotype, WT or *amiR23* and treatment, control or salinity) into a single factor that was used in a simple design formula (as recommended by the package vignette as alternative to more complex models).

198 Sequencing data were deposited at the European Nucleotide Archive
199 (<https://www.ebi.ac.uk/ena/>) with the accession id PRJEB47043.

200

201 Statistical analyses

202 The data shown in 1, 2, 3, and S5 were analyzed using a two-way ANOVA considering
203 genotype and treatment. When interaction terms were significant ($P < 0.01$),
204 differences between means were analyzed using Tukey comparison and are indicated
205 by different letters. The number of biological replicates for each assessment are
206 indicated in the corresponding figures.

207 The Venn diagram in Fig. 4A was performed using the R package VennDiagram (Chen,
208 2018) and the heatmap in Fig. 4B was done with the pheatmap package (Kolde, 2019).

209

210 Accession numbers

211 AT1G26960 (AtHB23); AT2G42430 (LBD16); AT1G77690 (LAX3)

212 Results

213 AtHB23 expression is regulated by NaCl strongly affecting root architecture

214 AtHB23 has been identified as a crucial player in LR initiation. This gene is a direct
 215 target of ARF7/19, regulated by salinity (Perotti *et al.*, 2019; <http://bar.utoronto.ca/>;
 216 Dinnery *et al.*, 2008). Since primary root and LR development are closely linked to soil
 217 conditions, we tested whether the role of AtHB23 was affected by salinity using NaCl.
 218 Root phenotype was evaluated in *AtHB23* overexpressors (AT23), knock-down
 219 (*amiR23*), and wild type (Col 0) 3-day-old plants treated for additional 5 days with
 220 different NaCl concentrations. Both silenced and overexpressors showed an altered
 221 phenotype compared to controls. The differences were more pronounced
 222 concomitantly with growing concentrations of NaCl. Knock-down plants exhibited a
 223 significantly reduced primary root length in 50 and 75 mM NaCl (Fig. 1A), whereas
 224 AT23 overexpressors showed the opposite phenotype in control conditions and 50 mM
 225 NaCl (Fig. 1E). When the concentration reached 75 mM, the difference between the
 226 WT and AT23 was undetectable (Fig. 1E). LRP density was higher in *amiR23* seedlings,
 227 in agreement with the previously reported results (Perotti *et al.*, 2019), but this
 228 difference with the WT diminished at increasing concentrations of NaCl (Fig. 1B). In
 229 contrast to control conditions, AT23 plants exhibited lower LRP density than in the WT
 230 in response to NaCl (Fig. 1F). LR density was larger in AT23 plants than in controls in 75
 231 mM NaCl, whereas silenced plants, at this NaCl concentration, behaved similarly to
 232 AT23 (Figs. 1C and 1G). Total LR followed the pattern of lateral root primordium (LRP),
 233 suggesting more influence on the LRP number than on LRs (Figs. 1D and 1H).
 234 To evaluate the expression pattern of *AtHB23* in salinity conditions, transgenic plants
 235 carrying the construct *prAtHB23_L:GUS* (1793 bp upstream from the start codon) were
 236 treated with NaCl and GUS activity visualized by histochemistry. In normal conditions,
 237 the promoter activity was restricted to the base of LRP at stages V and VI and
 238 disappeared when the LR emerged, whereas in advanced stages of emerged roots, a
 239 signal in the vascular system of both the main and LR was observed (Fig. S1A). After
 240 treating the roots with NaCl, GUS staining was restricted to the LRP and disappeared or
 241 diminished from the vascular system of emerged roots (Fig. S1B).
 242 Given the altered phenotype observed in plants with reduced (*amiR23*) or augmented
 243 (AT23) levels of *AtHB23*, we wondered how this TF was affecting the development of

the main root when its expression was not detectable in the root tip. Regulatory elements can be found in various locations, near or far away from coding sequences. Considering that the promoter region we isolated was arbitrarily defined as the 1793 bases upstream of the start codon (Perotti *et al.*, 2019), we decided to dissect the *AtHB23* promoter region to uncover its potential activity in the root tip. To this end, we cloned shorter segments fused to the reporter gene *GUS* and transformed WT plants. Interestingly, a segment of 1273 bp showed a strong activity in the tips of the main and secondary roots, and in the emerged LR (Figs. 1I and S2). Since this segment was 520 bp shorter than the one previously assessed (Perotti *et al.*, 2019, 2020 and Fig. S1), our results suggest that the deleted region might include a repressor box. Remarkably, when these plants were subjected to NaCl treatments, GUS staining was significantly increased in the tip of the main root, in recently emerged LR, as well as in the tip of secondary LR (Figs. 1J and S2). Therefore, the expression pattern of the shorter *AtHB23* promoter in the root tip in response to NaCl was in agreement with the differential root growth observed in *AtHB23*-deregulated plants.

259

260 Long treatments at increasing NaCl concentrations have a severe effect on *amiR23*- 261 silenced plants

To further characterize root architecture in salinity conditions, seedlings were subjected to increasing times and NaCl concentrations. The difference between WT, AT23, and *amiR23*-silenced plants in primary root length slightly increased between 4 and 10 days after sowing (Fig. 2A). However, the difference in the main root growth became more evident when seedlings were treated with NaCl. In 25 mM NaCl, the difference between genotypes augmented significantly (Fig. 2B) whereas, in 50 and 75 mM NaCl, *amiR23* plants almost arrested their development while AT23 and WT seedlings maintained a similar growth rate with a slight advantage for AT23 (Figs. 2C and 2D). Notably, the primary roots of *amiR23* seedlings showed a curved shape, suggesting that the gravitropic response was affected (Fig. 2).

Strikingly, the assessment of the survival rate of the different *AtHB23* knock-down and overexpressor lines at 100 mM NaCl for 6 and 15 days revealed a boosted sensitivity of *amiR23* plants to high salinity. After 6 days of treatment, 60 % of *amiR23* plants died, whereas all WT and AT23 plants survived. After 15 days, a growing percentage of WT

276 plants died whereas most of AT23 plants still survived, in contrast to the low survival
277 rate of *amiR23* plants (Figs. 2E and 2F).

278

279 The *amiR23*-silenced plants lost the starch granules in the root tip after salinity
280 treatment

281 To better characterize the high sensitivity of *amiR23* plants to NaCl, we grew them
282 together with the WT and AT23 genotypes during five days in normal conditions. Then,
283 seedlings were transferred to the same medium containing 150 mM NaCl for eight
284 additional hours. Afterwards, a group of plants was transferred back to MS medium to
285 recover, while another group remained in the NaCl-containing medium. From those
286 plants kept in NaCl, notably WT and AT23 slowly adapted and recovered similarly to
287 those transferred to the NaCl-free MS medium. On the other hand, the *amiR23* plants
288 kept in NaCl died. The roots of plants from all groups were treated with Lugol staining
289 solution to visualize starch in their root tips. In normal conditions, AT23 plants
290 exhibited more starch than the WT, whereas the *amiR23*-silenced plants had
291 significantly less starch than the controls (Figs. 3A and 3C). After NaCl treatment,
292 starch accumulation was affected in all the genotypes but AT23 plants showed a better
293 performance than control plants, whereas it was not possible to visualize the stained
294 polyglucan in *amiR23* tips (Figs. 3A and 3C). Notably, after recovery in a normal
295 medium, both WT and AT23 roots exhibited restored amyloplasts, whereas *amiR23*-
296 silenced plants did not recover and continued to lack visible starch (Fig. 3A). The same
297 experiment was carried out with independent lines of *amiR23*-silenced plants showing
298 similar characteristics to those of *amiR23-1*. Moreover, the phenotype of *amiR23*-
299 silenced plants was rescued by crossing them with AT23 plants (*amiR23* x AT23
300 genotype), restoring the ability to recover from NaCl treatment (Fig. S4).

301 To elucidate whether *amiR23* plants lost starch or if the amyloplasts were degraded,
302 resulting in starch dispersion, then not visible under the optic microscope, a
303 transmission electronic microscopy (TEM) was used to observe the treated roots. TEM
304 images indicated that *amiR23* plants neither had amyloplasts nor disseminated starch
305 after the NaCl treatment (Fig. 3B). Moreover, amyloplasts did not reappear after
306 transferring back the plants to a normal medium (Fig. 3B).

307 Altogether, our results indicate that AtHB23 is a key factor participating in the
308 adaptation of Arabidopsis plants to high salinity, notably assuring starch turnover
309 and/or amyloplast biogenesis.

310

311 Starch synthesis and degradation are significantly affected by AtHB23

312 We then wondered if starch biosynthesis, its degradation, or both were affected upon
313 *AtHB23* deregulation. To this end, we evaluated key genes involved in the pathways of
314 starch biosynthesis and degradation in AT23 and *amiR23* silenced plants (Fig. 3D).
315 Collectively, our results showed an accumulation of transcripts encoding enzymes
316 participating in starch degradation and reduced levels of transcripts encoding enzymes
317 necessary for the synthesis of the polyglucan (Figs. 3E and F). For example, *GWD* and
318 *BAM1* encoding the enzymes degrading starch to maltose and glucose (Fig. 3D), were
319 strongly induced in *amiR23* roots subjected to NaCl for 8 h (Fig. 3E). In agreement with
320 this, *PGM* and *ADG1* involved in starch biosynthesis (Fig. 3D), were induced in all
321 genotypes after recovery, but significantly more in WT and AT23 plants than in the
322 *amiR23* silenced plants (Fig. 3F). Other genes, also involved in starch synthesis and
323 degradation, showed significant changes in their transcripts depending on *AtHB23*
324 levels. For example, *SS2*, *SS3*, *SS4*, and *GBSS1*, which participate in the pathway of
325 starch synthesis (Fig. 3D), were significantly induced after recovery from NaCl
326 treatment in MS medium but such increase was lower in the silenced plants (Fig. S5).
327 Differently, *SS1* was strongly induced in the *amiR23* silenced plants after NaCl
328 treatment and showed an upregulation tendency in the WT, whereas in the AT23
329 genotype the differences were not significant (Fig. S5). Finally, *AMY3* and *BAM3*,
330 involved in starch degradation (Fig. 3D), were upregulated after recovery in all the
331 genotypes but to a lesser extent in the silenced plants (Fig. S5). Altogether, these
332 results indicated that starch turnover was significantly altered by the levels of *AtHB23*.
333 However, this fact solely cannot explain the absolute lack of starch observed in *amiR23*
334 plants, indicating that other mechanisms must be modulating this process.

335

336 A transcriptome analysis strongly supported the role of AtHB23 in carbohydrate 337 turnover

338 Because of the crucial role played by AtHB23 in salinity conditions, we wondered
339 which other signal transduction pathways, aside from starch turnover, could be
340 modulated by this TF. To further investigate this, an RNA-Seq transcriptomic
341 experiment was carried out using 9-day-old WT and *amiR23*-silenced plants grown in
342 control conditions or treated with 75 mM NaCl. In contrast to the analysis performed
343 for a subset of genes in response to short treatments with NaCl, this transcriptomic
344 approach served to uncover the AtHB23-dependent transcriptional reprogramming
345 during the plant adaptation to salinity. In control conditions, only 24 genes significantly
346 changed their expression in *amiR23* versus WT plants (Fig. 4A). Interestingly, the
347 number of differentially expressed genes (DEGs) between genotypes increased to 2025
348 in salinity conditions (Fig. 4A), comparing to control conditions, the salinity treatment
349 resulted in 2775 DEGs in WT plants and 4225 DEGs in *amiR23* plants. Thus, AtHB23
350 appears as a central regulator of the adaptation to long-term stress.

351 An analysis performed with the Mapman software allowed the identification of the
352 pathways in which DEGs were involved.

353 We first compared results obtained for *amiR23*-silenced plants treated with NaCl
354 versus control conditions. There were major changes in transcripts related to lipids,
355 secondary metabolism and cell wall (Figs. 4B and S6A). Some of these were severely
356 reduced, such as *EXTENSIN18* and *EXTENSIN19*, which encode hydroxyproline-rich
357 proteins putatively involved in cell wall assembly. Also, several transcripts encoding
358 different cellulose synthase isoforms (*CESA3*, *CESA4*, and *CESA8*) were decreased (Fig.
359 4B and Table S2). Conversely, some transcripts encoding extensins (which mediate cell
360 wall loosening) were highly increased, including *EXPANSIN1*, *EXPANSIN10*,
361 *EXPANSIN12*, and *EXPANSINB2* (Fig. 4B and Table S2). Intriguingly, there was a massive
362 increase of transcripts encoding components of the light reactions, tetrapyrrole
363 metabolism and the Benson-Calvin cycle (Fig. S6A). Additionally, we found a large
364 number of DEGs related to other processes, including hormone metabolism, response
365 to abiotic stress, cell cycle and protein degradation. Among these, we noticed
366 decreased levels of *ATG8A* and *ATG8I*, which encode components of the autophagy
367 conjugation pathway (Fig. 4B and Table S2). Also, multiple transcripts related to auxin
368 metabolism were altered. Particularly, *PIN5* (encoding an auxin transporter that
369 controls auxin levels in the lateral root cap) was decreased, while other transcripts

370 encoding the auxin carriers *PIN1*, *PIN3*, and *PIN7* were increased; intriguingly, the *LAX3*
371 transcript was also increased (Fig. 4B and Table S2).

372 Considering the lack of starch observed in *amiR23*-silenced plants treated with NaCl,
373 we focused our attention on DEGs related to starch and sucrose metabolism. The
374 transcripts *PGSIP1* and *PGSIP4*, encoding glycogenin-like proteins putatively involved in
375 the initiation of the starch granule, were reduced. Conversely, three transcripts
376 encoding different ADP-glucose pyrophosphorylase subunits (*APS1*, *APL3* and *APL4*)
377 and the *GBSS1* transcript (encoding a granule-bound starch synthase) were increased
378 (Fig. 4B and Table S2). We also found that several transcripts encoding enzymes
379 involved in starch degradation were increased, such as *GWD* (or *SEX1*, encoding a
380 glucan, water dikinase) and *SEX4* (encoding a glucan phosphatase), while *BAM9*
381 (encoding a beta-amylase) was decreased (Fig. 4B and Table S2). Transcripts related to
382 sucrose degradation showed different patterns. For instance, *CINV1* and *BFRUCT3*,
383 encoding cytosolic and vacuolar invertases, respectively, were reduced. Conversely,
384 the transcripts encoding the mitochondrial alkaline/neutral invertase C and the cell
385 wall invertase 5 (*CWINV5*) were increased, as well as those encoding sucrose synthases
386 1 and 3 (*SUS1* and *SUS3*, respectively; Fig. 4B and Table S2).

387 We then compared the results obtained with *amiR23*-silenced and WT plants treated
388 with NaCl (Fig. S6B). In this case, the changes were less prominent than in the previous
389 analysis (i.e. *amiR23* silenced plants treated with NaCl versus control conditions).
390 These results indicated that some responses to NaCl occurring in WT plants were
391 conserved in *amiR23* silenced plants and were consistent with qPCR data presented in
392 Figs. 3E and 3F. In general, we found DEGs involved in multiple cellular processes,
393 including response to abiotic stress, development, hormone metabolism and protein
394 degradation (Fig. 4B). Some of these transcripts were already observed in the previous
395 comparison. For example, reduced levels of transcripts encoding auxin transporters
396 (*PIN5* and *PIN6*) and increased levels of *ATG8E* (a component of the autophagy
397 pathway). Interestingly, transcripts involved in sucrose and starch metabolism showed
398 the same trend: *CINV1*, *BFRUCT3* and *BAM9* were reduced, while that encoding the
399 mitochondrial alkaline/neutral invertase C was increased (Fig. 4B and Table S2).

400

401 *amiR23*-silenced plants cannot recover from NaCl treatment either with auxin or
402 sucrose

403 Considering that auxin indirectly promotes the formation of starch granules (Zhang *et*
404 *al.*, 2019), we wondered whether NaCl seriously affecting *amiR23*-silenced plants can
405 be balanced by the effect of auxin. To test this hypothesis, *amiR23*, AT23 and WT
406 plants were grown in normal conditions for five days and then placed in NaCl for 8 h.
407 As previously shown, this treatment reduced starch content in the three genotypes
408 (Fig. 3). The addition of 1 μ M IAA (instead of 150 mM NaCl) to the MS medium during 8
409 h did not affect the scenario observed in control conditions (Fig. 5C), whereas the
410 addition of both 1 μ M IAA and 150 mM NaCl failed to counter the NaCl effect (Fig. 5D).
411 When the treatment with 150 mM was prolonged for 72 h, both WT and AT23 plants
412 recovered their starch, while *amiR23* plants remained affected (Fig. 5E). The treatment
413 with 1 μ M IAA during 72 h did not change the scenario of untreated plants (Fig. 5F),
414 and when both compounds were added together, control and AT23 plants showed
415 starch whereas *amiR23* plants irreversibly lost it (Fig. 5G). The same differences
416 between genotypes were observed when roots were placed in 150 mM NaCl for 8 h
417 and then placed in 1 μ M IAA or 1% sucrose for additional 72 h (Figs. 5H and 5H). These
418 results indicated that *amiR23* plants are incapable of recovering from the salinity
419 treatment; therefore AtHB23 plays a crucial in the response to this stress.

420

421 The AtHB23-target gene *LAX3* modulates the main root development in response to
422 NaCl

423 Considering that *LAX3* and *LBD16* genes were reported as direct targets of AtHB23 in
424 the context of LR development (Perotti *et al.*, 2019), we wondered whether these
425 genes were involved in the response to salinity. Transgenic plants bearing the
426 promoters of *LAX3* and *LBD16* fused to the *GUS* reporter gene were analyzed in control
427 conditions and after treatments with 75 mM NaCl in the main, secondary, and tertiary
428 roots. That is because the modulation of these genes was found to be dependent on
429 the root order (Perotti *et al.*, 2020). *LAX3* was expressed in the root tip of the main,
430 lateral, and third order roots in 14-day-old seedlings (Fig. 6A), whereas *LBD16* was
431 expressed in the tip of LR and third order root but not in that of the main root even
432 after NaCl treatment (Fig. S7). Surprisingly, after NaCl treatment, the signal

433 corresponding to *LAX3* only disappeared from the tip of the main root, remaining
 434 without changes in secondary and tertiary root tips (Fig. 6A). Moreover, transcript
 435 levels of *LAX3* measured after the treatment showed a marked reduction, in
 436 agreement with histological observations (Fig. 6B). Thus, we compared by histological
 437 observation 10-day-old *prLAX3:GUS* and crossed *prLAX3:GUS* x *amiR23-1* seedlings.
 438 Unexpectedly, *LAX3* signal disappeared from the main root tip of crossed plants, either
 439 treated or untreated (Fig. 6C), hinting at an AtHB23-mediated activation of *LAX3*
 440 transcription in the main root. These results strongly suggested that the repression
 441 exerted by NaCl on *LAX3* in the main root tip is stronger than the induction promoted
 442 by AtHB23, at least in this specific developmental stage and condition.
 443 Then, we wondered whether *LAX3* could alter the root phenotype in NaCl conditions.
 444 To this end, *lax3* mutant plants were evaluated after treatments with different NaCl
 445 concentrations, ranging from 0 to 75 mM. In control conditions, *lax3* mutants had
 446 slightly longer main roots than WT plants, and this difference increased in 25 and 50
 447 mM NaCl (analyzed for 10 days; Fig. 6D). On the other hand, the amount of starch in
 448 *lax3* mutant seedlings did not show significant differences with the WT plants, either
 449 under control conditions or after the salinity treatment (Fig. 6E). Altogether, these
 450 observations indicated that exposed to salinity, *LAX3* does not play a crucial role and is
 451 probably modulated by different TFs.

452 Discussion

453 Root developmental plasticity determines the capacity of plants to adapt to different
454 soil types. Among many characteristics defining soil quality, salinity emerges as a
455 strong feature affecting plant growth. Moreover, salinity depends on water availability,
456 continuously challenging roots to display their skills to capture water and
457 micronutrients.

458 Root architecture plasticity involves the development of the main, lateral, and roots of
459 higher order. The development of the main and lateral roots often exhibit opposite
460 responses involving different, sometimes contrary, growth patterns. For example,
461 when subjected to water deficit, the main root grows deeper in order to reach water,
462 and LR arrest their development (Julkowska and Testerink, 2015).

463 Under control conditions, root development is governed antagonistically by auxin and
464 cytokinin, but cell division and LR morphogenesis rely, in different ways, on auxin
465 signaling (Petricka *et al.*, 2012). For example, in the *diageotropica/wol* mutants
466 (tomato and Arabidopsis) LR development is arrested, and auxin cannot rescue the
467 phenotype (Parizot *et al.*, 2008; Ivanchenko *et al.*, 2006). It has been reported that
468 mild salinity enhances the growth of the main and LR, while NaCl increasing levels are
469 detrimental for both, indicating a salt concentration dependency of root growth (Zolla
470 *et al.*, 2010; Julkowska *et al.*, 2014a). However, in the Col 0 accession, the
471 development of the main root is more affected by salinity than that of LR and depends
472 on the exposure time (Julkowska *et al.*, 2014b). Moreover, it seems that the best
473 strategy for roots to survive salinity is to have few but longer LR to exclude Na⁺
474 (Julkowska and Testerink, 2015).

475 In this work, through the study of the homeodomain-leucine zipper transcription
476 factor AtHB23, we exemplify the significant changes that take place in the Arabidopsis
477 genetic program to deal with salinity stress. Previously, it was shown that in normal
478 growth conditions, the knock-down of *AtHB23* provoked an enhancement in LR
479 initiation. This TF is upregulated by ARF7/19, induced the expression of *LAX3* and
480 repressed that of *LBD16* in LR, and, importantly, this genetic program differed between
481 second and third-order roots (Perotti *et al.*, 2019, 2020). Under salinity stress, plants
482 with altered levels of *AtHB23* exhibited significant changes in root architecture. The LR
483 phenotype of *amiR23* silenced and AT23 plants was in accordance with the regulation

484 by NaCl (Figs. 1 and 2). Surprisingly, the main root of *AtHB23* knocked-down plants was
485 more affected than LR although we did not detect *AtHB23* expression. Remarkably, the
486 main root arrest, observed in *amiR23* plants, correlates with a compromised survival
487 under high salinity (Fig. 2). The analysis of plants transformed with a shorter segment
488 of the promoter region fused to *GUS* revealed a strong expression in columella cells
489 and an induction by NaCl both in the main and LR, explaining the differential
490 phenotype (Fig. 1). In a salinity environment, *AtHB23*-silenced plants showed arrested
491 growth of the main root and an increment in LRP, stronger than in normal media. The
492 opposite phenotype was exhibited in *AtHB23* overexpressor plants. Interestingly, in
493 the model legume *Medicago truncatula*, the HD-Zip I TF Mthb1 was shown to block LR
494 emergence in response to high salinity by directly repressing the expression of the
495 auxin-responsive LBD TF-encoding gene *MtLBD1*. Moreover, the main root tip of
496 *mthb1* mutants experienced a severe disorganization under stress (Ariel *et al.*, 2010a
497 and b). Although the closest homologs of Mthb1 in Arabidopsis are AtHB7 and 12, our
498 results indicate that AtHB23 and Mthb1 may exert similar roles in these distant
499 species.

500 One of the characteristics observed was the waiving of *amiR23* roots, indicating a loss
501 of the gravitropic response. Gravitropism is a crucial process allowing roots to grow
502 downwards and potentially capturing more water. This phenomenon involves the
503 accumulation of starch granules as statoliths that perceive gravity. This important
504 route is mediated by auxin and affected by salinity conditions (Zhang *et al.*, 2019).
505 Although some steps of this process were already described (Iglesias *et al.*, 2010), the
506 relationship between salinity, auxin, starch biosynthesis and degradation, and gravity
507 perception remains largely unknown. In normal growth conditions, PIN directional
508 auxin transporters generate an auxin gradient within the root apex, altering the
509 expression of *SS4*, *PGM*, and *ADG1*, genes encoding enzymes involved in the synthesis
510 of starch granules (Zhang *et al.*, 2019).

511 Degradation of amyloplasts in the columella cells was observed in water-stressed
512 roots, provoked by sorbitol or mannitol (Takahashi *et al.*, 2003). The silencing of
513 *AtHB23* resulted in the irreversible loss of starch in the columella cells of the main root
514 in response to NaCl, indicating a crucial role for this TF in the salinity response (Fig. 3).

515 The RNA-Seq data for *amiR23*-silenced plants treated with NaCl showed reduced levels
516 of transcripts encoding different isoforms of extensins and components of the
517 cellulose synthase complex, whereas transcripts encoding several expansins were
518 increased (Figs. 4 and S6). These results suggest altered turnover of the cell wall
519 (reduced synthesis and increased degradation), which could affect its integrity, thus
520 increasing the susceptibility to NaCl (Liu *et al.*, 2021). These observations were in
521 accordance with the low survival rate exhibited by *amiR23*-silenced plants after NaCl
522 treatment (Fig. 2).

523 In agreement with the observed phenotype, starch turnover was severely affected by
524 AtHB23. This was observed not only by individual evaluation of transcripts by RT-qPCR,
525 but also by the transcriptome analysis. Transcripts encoding starch-synthesizing
526 enzymes were decreased, while those encoding starch-degrading enzymes were
527 increased (Figs. 3 and S5). Intriguingly, *ADG1* and *GBSS1* were increased in the RNA-
528 Seq when comparing *amiR23*-silenced plants in control versus salinity conditions (Figs.
529 4 and S6). These results seem counterintuitive; however, it must be considered that
530 *ADG1* and *GBSS1* were induced in *amiR23*-silenced plants treated with NaCl, but to a
531 lesser extent than in WT and AT23 plants (Figs. 3F and S5). Consequently, these
532 transcripts were not found in the RNA-Seq as DEGS in salinity conditions (*amiR23*
533 versus WT; Figs. 4 and S6). We also noticed reduced levels of the transcripts *PGSIP1*
534 and *PGSIP4* in *amiR23* plants treated with NaCl compared with control conditions (Figs.
535 4 and 6). Interestingly, Arabidopsis plants expressing a *PGSIP1* RNAi construct showed
536 reduced levels of starch in leaves (Chatterjee *et al.*, 2005), which could explain the
537 starchless phenotype of *amiR23* plants treated with NaCl.

538 Sucrose degradation is catalyzed by sucrose synthases or invertases (Barratt *et al.*,
539 2009). Arabidopsis has six genes encoding cytosolic (*SUS1-4*) and vascular (*SUS5-6*)
540 sucrose synthases and multiple genes coding for cytosolic, vacuolar, and apoplastic
541 invertases. The *sus1/sus2/sus3/sus4* quadruple mutant showed normal contents of
542 sucrose and starch, while the *sus5/sus6* double mutant exhibited reduced callose.
543 Notably, when the genes encoding the main cytosolic invertase isoforms in roots
544 (*CINV1/CINV2*) were mutated, plants exhibited a severely affected phenotype,
545 including loss of starch in the columella cells (Barratt *et al.*, 2009; Pignocchi *et al.*,
546 2021). Although the altered morphology of *cinv1/cinv2* mutants was similar to that

547 observed in *amiR23* silenced plants treated with NaCl, transcript levels of *CINV1* and
548 *CINV2* did not significantly change in *amiR23* plants exposed to 150 mM NaCl for 8 h,
549 suggesting that *AtHB23* is not regulating sucrose degradation through this pathway.
550 However, we observed decreased levels of the *CINV1* transcript in *amiR23*-silenced
551 plants exposed to 75 mM NaCl for 6 days (Figs. 4 and S6).

552 Vacuolar invertases are involved in the regulation of plant growth and development in
553 response to abiotic stress. The tea *INV5* was reported as induced by cold and
554 carbohydrates; the overexpression of this gene promoted taproot and LR elongation
555 involving carbohydrates and auxin signaling (Qian *et al.*, 2018). Interestingly, we found
556 consistently reduced levels of the *BFRUCT3* transcript, encoding the vacuolar invertase
557 1 (Sergeeva *et al.*, 2006), in *amiR23* plants (Figs. 4 and S6).

558 Notably, and considering that auxin promotes starch granule formation, the hormone
559 did not change the amount of starch in columella cells when roots were treated alone
560 neither rescued starch degradation in *amiR23* plants, even after 72 h. Such plants
561 irreversibly lost their starch granules (Fig. 5). These results are in good agreement with
562 those reported by Pignocchi *et al.* (2021), who showed that disruption of auxin
563 signaling does not explain the phenotype of the *cin1/cin2* double mutant (see
564 above).

565 Auxin homeostasis emerged also as an additional factor linked to *AtHB23*-mediated
566 response to salinity. *LAX3*, previously shown to be directly regulated by *AtHB23*
567 (Perotti *et al.*, 2019), was repressed by 75 mM NaCl in the main root tip, but not in the
568 tips of higher-order roots (Fig. 6A). While the *LAX3* signal disappeared in the root tip of
569 *prLAX3:GUS* x *amiR23-1* crosses, indicating a positive regulation by *AtHB23*,
570 unexpectedly, NaCl repressed *LAX3* expression (Fig. 6C). This puzzling scenario cannot
571 be easily explained. We can hypothesize that although *AtHB23* induces *LAX3*, and the
572 former is upregulated by 75 mM NaCl, the impact of salinity overpowers that of this
573 TF. A possible pathway for such effect could be the upregulation of another/unknown
574 HD-Zip I TF, able to heterodimerize with *AtHB23*, avoiding its action on *LAX3*. In this
575 sense, HD-Zip I TFs are able to heterodimerize (Capella *et al.*, 2014), and several
576 members are expressed in the columella cells (Perotti *et al.*, 2021). An alternative
577 explanation could be supported on NaCl concentration and treatment time-
578 dependency of the response. *LAX3* expression was repressed in 14-day old crossed

579 plants and in 5-day-old plants treated for 8 h with 150 mM NaCl (Fig. 6C), whereas in
 580 the transcriptome analysis, performed with 9-day-old plants subjected for 6 days to a
 581 lower salt concentration (75 mM), this gene appeared slightly induced (Table S2).
 582 Altogether, the results shown here indicated that AtHB23 plays opposite roles in the
 583 main and LR when plants were subjected to salinity and that it is necessary for plant
 584 survival under stress (Fig. 7). This TF is required to recover starch biosynthesis after
 585 NaCl treatment in order to form granules, necessary to sense and respond to gravity.
 586 AtHB23 integrates the modulation of genes participating both in starch turnover and
 587 auxin transport.

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596

597 **Conflict of interest**

598 The authors declare no competing interests.

599

600 **Authors' contribution**

601 Conceived the experiments: MFP, CMF, FDA, and RLC. MFP performed most of the
602 experiments. ALA performed the bioinformatics analyses and wrote the corresponding
603 items. Conceived and wrote the paper: RLC. All authors revised, discussed, and
604 approved the manuscript.

605

606 **Data availability**

607 All the data and materials that support the findings of this study are available upon
608 request from the corresponding author.

609

610 **Supporting information**

611 Additional Supporting Information may be found in the online version of this article.

612 **Table S1: oligonucleotides used in this work**

613 **Table S2: Transcriptomic analysis of wild type and *amiR23* plants in control and**
614 **salinity conditions**

615 **Fig. S1. The expression pattern of *AtHB23* is modulated by NaCl in lateral roots**

616 **Fig. S2. *AtHB23* expression is induced by salinity conditions in the main and lateral**
617 **roots**

618 **Fig. S3. *AtHB23* levels affect main root phenotypes**

- 619 **Fig. S4. Starch loss in independent *amiR23*-silenced lines under salinity stress is**
 620 **rescued in *amiR23*×*AT23* plants**
- 621 **Fig. S5. Transcript levels of genes involved in starch metabolism turnover**
- 622 **Fig. S6. Mapman analysis of the DEGs *amiR23* plants compared with Col 0 in normal**
 623 **or salinity conditions**
- 624 **Fig. S7. *LBD16* expression is not altered by salinity conditions**

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Legends to figures

Figure 1. The expression pattern of *AtHB23* is differentially affected by NaCl in the main and lateral roots, altering whole root development

(A-D) Relative primary root length (A) of 8-day-old Col 0 and *amiR23-1* silenced plants, relative density of lateral root primordium (LRP, B), lateral root (LR, c), and total LR (LRP + LR, D) calculated as the number of LRP or LR/mm of main primary root, grown in control conditions during 8 days (control) or 3 days and treated with NaCl (50 or 75 mM), during 5 additional days. (E-H) The same as in (A-D) evaluated in Col 0 or AT23 (*AtHB23* overexpressor) plants. The values were normalized with those measured in the Col 0 control, taken as 1 (one). All the assays were repeated at least three times with N: 15/genotype. (I) Illustrative pictures showing GUS expression driven by *AtHB23* promoter (short version, *prAtHB23₅:GUS*) in the tips of main and lateral roots of 8-day-old seedlings grown in control conditions. (J) The same as in (I) in plants grown 3 days in normal conditions and then placed during 5 additional days in 75 mM NaCl. LR are emerged lateral roots. Black bar represents 50 μ m.

Figure 2. *AtHB23* promoted main root elongation under salinity stress

(A) Main root length evaluated in Col 0, AT23-1 (overexpressors), and *amiR23-1* (silenced) seedlings grown in normal conditions. In the left panel quantitative measurements performed from day 4 after sowing and until day 10, and in the right panel, illustrative pictures of the same roots taken the 10th day. The same plants as in (A) were transferred 3 days after sowing to plates supplemented with 25 mM (B), 50 mM (C) or 75 mM (D) NaCl. The assays were repeated at least three times with N: 15/genotype. Black bar represents 1 cm. Different letters indicate significant differences (Tukey test, $P < 0.01$). Error bars represent SEM.

(E-F) Survival rate of plants (Col 0, AT23 and *amiR23*) placed in plates with 100 mM NaCl, 3 days after sowing during the 6 (E), and 15 (F) additional days. Yellow columns indicate the % of dead plants whereas green ones the % of survivors. The assays were repeated at least three times with N: 15/genotype.

Figure 3. The amyloplasts in the root tip of *amiR23* silenced plants were degraded in NaCl medium and were unable to recover

(A) From top to bottom, illustrative pictures of root tips (5-day-old) stained with Lugol solution of Col 0, AT23, and *amiR23* seedlings grown in normal conditions (top), after 8 (upper-middle) or 72 h (bottom) treatment with 150 mM NaCl, and then transferred to normal conditions for additional 72 h (lower-middle). Black bar represents 50 μ m. (B) Illustrative pictures taken with Transmission electron microscopy (TEM) of root tips of 8-day-old seedlings grown in normal conditions (top), treated during 8 h with NaCl (middle), and placed again in normal conditions for 72 h additional hours (bottom). White bars represent 2 μ m. (C) Starch quantification by ImageJ software in the roots treated or not with 150 mM NaCl. (D) Schematic representation of the metabolic pathways for starch synthesis or degradation. (E-F) Transcript levels of key genes in Col 0, AT23-1, and *amiR23-1* plants grown in normal conditions during 5 days, treated 8 h with 150 mM NaCl and placed to recover in MS medium during additional 72 h. Genes assessed participating from degradation were *GWD* and *BAM1* (E), and from synthesis *PGM*, and *ADG1*. All the values were normalized with the one obtained in Col 0. Bars represent SEM. Data were analyzed using a two-way ANOVA considering genotype and treatment. Different letters indicate significant differences (Tukey test, $P < 0.01$). The accession numbers of the tested genes are the following: AT5G48300 (*ADG1*), AT5G51820 (*PGM*), AT1G10760 (*GWD*), AT3G23920 (*BAM1*).

Figure 4. Transcriptome analyses indicated a differential regulation in *amiR23* roots of genes involved and starch metabolism under salinity

(A) Venn Diagram showing the overlap of the sets of differentially expressed genes (FDR-adjusted p-value < 0.05) in the four different contrasts analyzed, in which the level of one factor is fixed and the two levels of the other factor are compared, e.g. the genotype is Col 0 and the comparison is performed between salinity and control conditions. (B) Heatmap showing the expression pattern of relevant genes (rows) in relation to the *amiR23* phenotype. They were selected based on the results of the Mapman analysis of DEGs (Fig. S6), and the inspection of the complete transcriptome (Table S2). The functional annotation of genes is highlighted on the left. Only the three contrasts with a relevant number of DEGs were included (columns).

Figure 5. Neither auxin nor sucrose were able to revert amyloplast degradation in *amiR23*-silenced plants

From top to bottom, illustrative pictures of root tips (5-day-old) stained with Lugol solution of Col 0, AT23, and *amiR23* seedlings grown in normal conditions (A), after 8 h treatment with 150 mM NaCl (B), or with 1 μ M IAA (C), or with 150 mM NaCl + 1 μ M IAA (D). (E) 5-day-old roots treated for 72 h with 150 mM NaCl, or with 1 μ M IAA (F), or with 150 mM NaCl + 1 μ M IAA (G), or treated during 8 h with 150 mM NaCl and then transferred to MS medium supplemented with 1 μ M IAA (H) or 1% sucrose (i) for additional 72 h. The assays were repeated at least three times with N: 15/genotype. Black bars represent 50 μ m.

Figure 6. The expression of the auxin carrier *LAX3* is repressed by AtHB23 under salinity stress in the main root but not in higher order roots

(A) Illustrative pictures showing GUS expression driven by *LAX3* promoter (*proLAX3:GUS*) in 14-day-old seedlings. Plants were grown in normal conditions for 3 days and then placed in MS plates (left panel) or MS supplemented with 75 mM NaCl (right panel) for 11 additional days. LR: lateral roots; TLR: tertiary roots. (B) Transcript levels of *LAX3* in 5-day-old seedlings grown in normal conditions (control) or treated with 150 mM NaCl for 8 h. The values were normalized with that obtained in normal conditions. Bars represent SEM. (C) GUS staining in primary roots of *prLAX3:GUS* or *prLAX3:GUS* x *amiR23* 10-day-old plants grown in normal conditions (left panel) or sowed in MS during 3 days and treated with 75 mM NaCl during 7 additional days (right panel). Black bars represent 100 μ m. (D) Relative main root length assessed in *lax3* mutants compared with the Col 0 controls, both treated with different NaCl concentrations. Bars represent SEM. Data were analyzed using a two-way ANOVA considering genotype and treatment. Different letters indicate significant differences (Tukey test, $P < 0.01$). (E) Illustrative pictures of Col 0 and *lax3* mutants root tips stained with Lugol in normal conditions or treated with 150 mM NaCl for 8 h.

Figure 7. Proposed model for AtHB23 in salinity conditions

Summary of the observations done, focused in the regulation by NaCl and auxin of main (A) and lateral (B) root development by AtHB23.

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A **B**

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