pH-dependent transcriptional profile changes in iron-deficient Arabidopsis roots

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Abstract

Background: Iron is an essential element for plants and abundantly present in most mineral soils. The mobility of iron, however, dependent on the redox potential and hydrogen activity (pH) of the soil, factors that may limit its availability to plants in particular at alkaline pHs. Iron deficiency triggers pronounced changes in the transcriptional profile of plants, inducing processes that aid in the acquisition, uptake, and translocation of iron. How ambient pH impact the transcriptional iron deficiency response has not yet been elucidated in detail.

Results: Here, we provide an RNA-seq data set that catalogs global gene expression changes of iron-deficient plants grown at either optimal (5.5) or high (7.0) pH. A suite of 857 genes changed significantly and more than twofold in expression; only 54 genes of this suite were also differentially expressed between iron-deficient and iron-sufficient plants grown at pH 5.5. Among the high pH-responsive genes, 186 were earlier shown to be responsive to short-term transfer to low pH, 91 genes of this subset were anti-directionally regulated by high and low pH. The latter subset contained genes involved in cell wall organization, auxin homeostasis, and potential hubs of yet undefined signaling circuits. Growing iron-deficient plants at high pH also modulated the transcriptional iron deficiency response observed at pH 5.5 by compromising the enzymatic reduction of ferric chelates and favoring the production of iron-mobilizing coumarins.

Conclusions: It is concluded that ambient pH is an important determinant of global gene expression which tunes iron acquisition to the prevailing edaphic conditions.

Keywords: Ambient pH, Coumarins, Iron deficiency, Iron uptake, RNA-seq, Transcriptome, Alkaline soil
Alkaline conditions, on the other hand, not only restrict the availability of iron, but also compromise the enzymatic reduction of ferric chelates by the plasma membrane-bound reductase FRO2, a central part of the iron uptake mechanism [27]. Restricted mobilization and limited uptake of iron constitute main factors for excluding so-called calcifuge (‘chalk-fleeing’) plants from habitats with alkaline soil reaction [31].

While the transcriptional response of Arabidopsis to iron starvation has been well explored (e.g. [10, 23, 28]), most studies were conducted at slightly acidic pH which is optimal for growth, but leaves the influence of alkaline pH on global gene expression profiles undefined. Knowledge on how ambient pH, in particular neutral or alkaline conditions, affects the transcriptional landscape of iron-deficient plants may aid in understanding the bottlenecks of gene regulation in plants that are not well adapted to soils with limited iron availability. Here, we provide an RNA-seq-based inventory of genome-wide gene expression of roots from iron-deficient Arabidopsis plants grown either at optimal, slightly acidic (5.5), or high (7.0) pH. To mimic the restricted availability of iron at high pH, we provided an iron source of low solubility to plants grown at neutral pH. Alkaline soil reaction aggravates iron deficiency symptoms, but the effect of pH per se has not yet been clearly distinguished from the response to a lack of iron at a hydrogen activity that is optimal for growth. We believe that the data set provided here allows for gaining valuable insights into the underlying causes of restricted iron uptake, limiting growth in natural or agronomical ecosystems with high pH, and sets the stage for follow up experimentation aimed at identifying novel players involved in the adaptation of plants to the prevailing hydrogen activity.

**Results**

**Ambient pH profoundly alters the transcriptomic profile of iron-deficient plants**

To investigate the influence of ambient pH on the global gene expression profile of iron-deficient plants, Arabidopsis seedlings were grown for 14 days on either optimal (5.5) or high (7.0) iron-deficient media, and roots were subjected to transcriptome profiling by RNA-seq. Approximately 24 million reads per treatment were captured during sequencing on the Illumina HiSeq 2000 platform, and mapped to the TAIR10 annotation of the Arabidopsis genome.

A flowchart of the experiment is depicted in Fig. 1a. After filtering lowly expressed genes which may not be of biological relevance (expression level in RPKM < the square root of the mean expression value of the whole dataset), a total of 857 genes were defined as being differentially expressed between iron-deficient plants grown at optimal or high pH media with a < 0.5 or > 2-fold change and a P value of < 0.05 (Fig. 1a). The expression of a suite of 37 differentially expressed genes (DEGs) was changed more than tenfold (Supplemental Table 1), indicative of robust alterations in transcriptional activity by a relatively subtle change in ambient pH.

To gain insights into the biological significance of the changes in gene expression, a gene ontology (GO) enrichment analysis for the DEGs was conducted using the Singular Enrichment Analysis (SEA) algorithm available on the AgriGO v2.0 toolkit [29], and visualized with the REVIGO Web server [26]. Categorizing the genes that are responsive to high pH under iron-deficient conditions revealed an overrepresentation of the GO categories ‘response to chemical’ and ‘response to stimulus’, indicating that the majority of pH-responsive genes is involved in adapting the plants to environmental conditions (Fig. 1b). Further, the GO category ‘secondary metabolism’ was significantly enriched. Surprisingly, also genes associated with photosynthesis were overrepresented in the data set, probably mirroring photosynthesis-related processes that are mainly affected in leaves where their expression is altered by high pH to avoid photo-oxidative damage.

Exposure to high pH modulated the iron deficiency response of the plants by altering the expression of several key genes functioning in the acquisition of iron. In high pH plants, FRO2 transcript levels were reduced by approximately 50% relative to plants grown at pH 5.5 (Supplemental Table 1), matching previous observations of severely compromised in vivo ferric reduction at high pH [27]. By contrast, expression of the 2-oxoglutarate-dependent oxygenase SCOPOLETIN 8-HYDROXYLASE (S8H) was more than threefold induced when plants were grown on high pH. S8H catalyzes the biosynthesis of the iron-mobilizing coumarin fraxetin (7,8-dihydroxy-6-methoxy-2H-chromen-2-one) which is secreted into the rhizosphere under iron-deficient conditions, particularly at high pH [19, 24, 25, 30]. Fraxetin mobilizes iron by both reduction and chelation, and may form stable complexes with Fe2+ [19, 30]. Fraxetin-mediated iron reduction is favored by mildly alkaline pH, compensating for the reduced enzymatic ferric reduction activity under such conditions. Interestingly, expression of another iron-responsive gene in the coumarin biosynthesis pathway, the cytochrome P450 CYP82C4, was completely abolished when plants were grown on high pH media (Supplemental Table 1). CYP82C4 catalyzes the hydroxylation of fraxetin to form sideretin (5,7,8-trihydroxy-6-methoxy-2H-chromen-2-one), a catecholic coumarin with a lower pH optimum for iron mobilization when compared to fraxetin [19]. It thus appears that ambient pH can modulate the activity of metabolic enzymes to prioritize the production of specific compounds in order to adapt iron acquisition to the prevailing edaphic
conditions. It is noteworthy that the activity of the two enzymes was altered at the transcriptional level, suggesting that ambient pH is sensed and relayed to the nucleus to modulate transcript abundance, possibly via recruitment of trans-acting factors. Such a putative pH sensing system has not been described in plants previously.

### Ambient pH modulates the iron deficiency response

A suite of circa 250 genes that are differentially expressed between iron-sufficient and iron-deficient plants surveyed at optimal pH constitute the ‘ferrome’, a robust shift in the transcriptional profile that induces various iron mobilization strategies and aids in recalibrating cellular iron homeostasis (e.g. [6, 10, 22]). To investigate the effect of a pH shift on iron-deficient plants, a comparative analysis was conducted between high pH-responsive DEGs and DEGs from an RNA-seq-based profiling of iron-sufficient and iron-deficient plants grown under conditions similar to that of the current study except that plants were grown on pH 5.5 media [22]. A comparison of the high pH-responsive DEGs with the ferrome revealed an overlap of 54 genes.
Genes from this overlap were mainly enriched in GO terms associated with 'ion homeostasis' and 'response to transition metal nanoparticle' (Fig. 2b). Interestingly, more than half of the genes are oppositely regulated between plants grown at optimal and high pH. Some 74% of the genes that were highly upregulated under iron-deficient conditions at optimal pH were downregulated when plants were grown on high pH media. This group included several key regulators of iron uptake including the subgroup Ib bHLH proteins bHLH39 and bHLH101 [33], suggesting a direct or indirect involvement of these proteins in the pH-dependent regulation of iron uptake (Fig. 2c). This subset also comprises the iron transporter IRT2 and the oxidoreductase FRO2, which catalyzes the reductive splitting of ferric chelates prior to uptake, the rate-limiting step of iron uptake [3, 7, 21, 32]. The reasons for this observation remain to be clarified. It may be speculated that the plant response to high pH interferes at some nodes with that to Fe deficiency, suggestive of a differential prioritization of different iron uptake strategies at different pH conditions.
Exposure of plants to different media pH uncovers putative nodes in pH signaling

A previously conducted transcriptional survey of *Arabidopsis* roots exposed to low (4.5) pH medium showed that the expression of a total of 1036 genes was significantly changed after transfer from pH 6.0 [14]. A comparison of this subset to our high pH data set revealed an overlap of 186 genes. In terms of GO categories, these common DEGs are enriched in the biological functions ‘response to chemical’, ‘immune system process’, ‘cell wall organization and biogenesis’, and ‘regulation of hormone levels’ (Fig. 3a). A group of 91 genes were anti-directionally regulated by high and low pH (Table 1). The latter list may contain genes that are involved in the perception and transduction of ambient pH. *PLANT INTRACELLULAR RAS GROUP-RELATED LRR 8* (PIRL8), a member of a novel class of plant-specific LRR proteins without clearly defined function [5], was found to be highly upregulated in response to high pH and strongly repressed by low pH (Table 1). Also, several genes involved in cell wall organization such as the pectin lyase At2g43890, the pectin methylesterase *ATPM*
Table 1 Genes with anti-directional regulation by low and high media pH. High pH data derived from the present study, low pH data are taken from Lager et al. [14]

| Locus          | Description                                                                 | High pH (1 h) | Low pH (8 h) | High/low pH ratio | Foldchange |
|----------------|------------------------------------------------------------------------------|---------------|--------------|-------------------|------------|
| AT2G41810      | Imidazolonepropionase                                                        | 13.56         | 0.06         | 214.45            |            |
| AT4G01630      | EXP17, expansin A17                                                           | 4.32          | 0.10         | 43.85             |            |
| AT1G43800      | SAD6, plant stearoyl-acyl-carrier-protein desaturase family protein          | 4.26          | 0.10         | 42.98             |            |
| AT1G09750      | Eukaryotic aspartyl protease family protein                                  | 2.68          | 0.07         | 39.09             |            |
| AT1G33840      | Protein of unknown function (DUF567)                                          | 10.88         | 0.28         | 38.25             |            |
| AT4G26050      | PIRL8, plant intracellular ras group-related LRR 8                           | 8.22          | 0.23         | 35.38             |            |
| AT1G50050      | CAP (Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 protein) superfamily protein | 5.69          | 0.20         | 28.49             |            |
| AT2G43890      | Pectin lyase-like superfamily protein                                        | 6.32          | 0.23         | 26.91             |            |
| AT5G48430      | Eukaryotic aspartyl protease family protein                                  | 6.61          | 0.30         | 21.76             |            |
| AT4G33560      | WIPS, wound-responsive family protein                                        | 4.06          | 0.27         | 15.26             |            |
| AT2G43620      | Chitinase family protein                                                     | 6.43          | 0.44         | 14.52             |            |
| AT1G16510      | SAUR41, SAUR-like auxin-responsive protein family                           | 3.94          | 0.28         | 14.13             |            |
| AT5G45340      | CYP707A3, cytochrome P450, family 707, subfamily A, polypeptide 3            | 3.15          | 0.23         | 13.62             |            |
| AT5G60770      | NRT24, nitrate transporter 2.4                                               | 5.60          | 0.42         | 13.30             |            |
| AT3G47380      | ATPMEL11, plant invertase/pectin methylesterase inhibitor superfamily protein | 4.07          | 0.31         | 13.00             |            |
| AT3G12830      | SAUR72, SAUR-like auxin-responsive protein family                           | 3.05          | 0.33         | 9.33              |            |
| AT4G10270      | WIP4, WOUND-RESPONSIVE FAMILY PROTEIN                                        | 3.41          | 0.46         | 7.37              |            |
| AT5G39890      | PCO2, PLANT CYSTEINE OXIDASE 2                                               | 2.88          | 0.43         | 6.78              |            |
| AT5G15230      | GASA4, GAST1 protein homolog 4                                               | 2.98          | 0.45         | 6.58              |            |
| AT1G05300      | ZIPS, zinc transporter 5 precursor                                           | 3.11          | 0.50         | 6.25              |            |
| AT3G10040      | HRA1, HYPOXIA RESPONSE ATTENUATOR1                                          | 3.07          | 0.49         | 6.19              |            |
| AT3G27220      | Galactose oxidase/kelch repeat superfamily protein                           | 2.77          | 0.45         | 6.11              |            |
| AT5G66460      | MAN7, glycosyl hydrolase superfamily protein                                | 2.18          | 0.43         | 5.07              |            |
| AT4G02290      | GH9B13, glycosyl hydrolase 9B1                                               | 2.35          | 0.47         | 4.99              |            |
| AT1G67750      | Pectate lyase family protein                                                 | 2.31          | 0.47         | 4.89              |            |
| AT1G70710      | CEL1, GH9B1, glycosyl hydrolase 9B1                                          | 2.24          | 0.49         | 4.57              |            |
| AT4G19230      | CYP707A1, cytochrome P450, family 707, subfamily A, polypeptide 1            | 2.14          | 0.48         | 4.44              |            |
| AT2G29330      | TRI, TROPINONE REDUCTASE                                                     | 0.49          | 2.28         | 0.22              |            |
| AT1G35260      | MLP165, MLP-LIKE PROTEIN 1                                                   | 0.47          | 2.26         | 0.21              |            |
| AT4G38470      | Dehydrin family protein                                                      | 0.47          | 2.31         | 0.20              |            |
| AT5G13750      | ZIFL1, ZINC INDUCED FACILITATOR-LIKE 1                                       | 0.38          | 2.05         | 0.18              |            |
| AT4G15610      | Uncharacterized protein family                                               | 0.45          | 2.45         | 0.18              |            |
| AT3G21690      | MATE efflux family protein                                                   | 0.41          | 2.31         | 0.18              |            |
| AT5G66170      | STR18, SULFURTRANSFERASE 1                                                   | 0.41          | 2.41         | 0.17              |            |
| AT1G13600      | BZIP58, BASIC LEUCINE-ZIPPER 58                                              | 0.45          | 2.65         | 0.17              |            |
| AT3G14990      | DJ1A, DJ-1 HOMOLOG A                                                        | 0.41          | 2.42         | 0.17              |            |
| AT1G65310      | XTH17, XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 17                          | 0.49          | 2.95         | 0.17              |            |
| AT2G32150      | Haloacid dehalogenase-like hydrolase (HAD) superfamily protein              | 0.49          | 3.13         | 0.16              |            |
| AT1G55920      | SAT1, SERINE ACETYLTRANSFERASE 1                                             | 0.36          | 2.56         | 0.14              |            |
Table 1 Genes with anti-directional regulation by low and high media pH. High pH data derived from the present study, low pH data are taken from Lager et al. [14] (Continued)

| Locus     | Description                                           | High pH (1 h) | Low pH (8 h) | High/low pH ratio | Foldchange |
|-----------|-------------------------------------------------------|---------------|--------------|-------------------|------------|
| AT2G39380 | EXO70H2, EXOCYST SUBUNIT EXO70 FAMILY PROTEIN H2     | 0.39          | 2.78         | 0.14              |            |
| AT4G38540 | FAD/NAD(P)-binding oxidoreductase family protein      | 0.40          | 2.88         | 0.14              |            |
| AT3G11340 | UGT76B1, UDP-DEPENDENT GLYCOSYLTRANSFERASE 76B1       | 0.34          | 2.47         | 0.14              |            |
| AT1G53990 | GLIP3, GDSL-MOTIF LIPASE 3                           | 0.31          | 2.27         | 0.14              |            |
| AT1G67810 | SUEF2, SULFUR E2                                      | 0.32          | 2.35         | 0.13              |            |
| AT1G55850 | CSL1, CELLULOSE SYNTHASE LIKE E1                      | 0.42          | 3.37         | 0.12              |            |
| AT5G37260 | CIR1, CIRCADIAN 1                                     | 0.32          | 2.63         | 0.12              |            |
| AT3G22370 | AOX1A, ALTERNATIVE OXIDASE 1A                         | 0.34          | 2.85         | 0.12              |            |
| AT1G01720 | ANAC2, ARABIDOPSIS NAC DOMAIN CONTAINING PROTEIN 2    | 0.46          | 4.12         | 0.11              |            |
| AT4G30670 | Putative membrane lipoprotein                         | 0.28          | 2.65         | 0.10              |            |
| AT1G43160 | RAP2.6, RELATED TO AP2 6                              | 0.33          | 3.21         | 0.10              |            |
| AT2G29490 | GSTU1, GLUTATHIONE S-TRANSFERASE TAU 1                | 0.24          | 2.32         | 0.10              |            |
| AT1G51420 | SPP1, SUCROSE-PHOSPHATASE 1                           | 0.41          | 4.06         | 0.10              |            |
| AT1G49570 | Peroxidase superfamily protein                        | 0.43          | 4.36         | 0.10              |            |
| AT1G77450 | ANAC032, NAC DOMAIN CONTAINING PROTEIN 32             | 0.27          | 2.91         | 0.09              |            |
| AT1G73260 | KTI1, KUNITZ TRYSIN INHIBITOR 1                       | 0.33          | 3.75         | 0.09              |            |
| AT1G80240 | DGR1, DUFS64 L-GALL RESPONSIVE GENE 1                 | 0.25          | 2.95         | 0.08              |            |
| AT3G46230 | HSP17.4, HEAT SHOCK PROTEIN 17.4                     | 0.17          | 2.03         | 0.08              |            |
| AT1G17180 | GSTU25, GLUTATHIONE S-TRANSFERASE TAU 25              | 0.17          | 2.18         | 0.08              |            |
| AT5G24090 | CHIA, CHITINASE A                                     | 0.29          | 3.94         | 0.07              |            |
| AT3G01420 | DOX1, PLANT ALPHA DIOXYGENASE 1                       | 0.25          | 3.53         | 0.07              |            |
| AT5G25460 | DGR2, DUFS64 L-GALL RESPONSIVE GENE 2                 | 0.38          | 5.47         | 0.07              |            |
| AT4G13180 | NAD(P)-binding Rossmann-fold superfamily protein      | 0.20          | 3.16         | 0.06              |            |
| AT2G29420 | ATGSTU7, GLUTATHIONE S-TRANSFERASE TAU 7              | 0.22          | 3.64         | 0.06              |            |
| AT5G61820 | Stress up-regulated Nod 19 protein                    | 0.17          | 2.96         | 0.06              |            |
| AT2G22860 | PSK2, PHOTOSULFOXINE 2 PRECURSOR                     | 0.23          | 4.08         | 0.06              |            |
| AT1G76520 | PILS3, PIN-LIKES 3                                   | 0.14          | 2.66         | 0.05              |            |
| AT5G50760 | SAUR55, SMALL AUXIN UPREGULATED RNA 5S                | 0.32          | 6.13         | 0.05              |            |
| AT1G05340 | ATHCSTM1, CYSTEINE-RICH TRANSMEMBRANE MODULE 1        | 0.40          | 8.31         | 0.05              |            |
| AT2G46750 | GULLO2, L-GULONO-1,4-LACTONE (L-GULL) OXIDASE 2       | 0.20          | 4.62         | 0.04              |            |
| AT1G78340 | GSTU22, GLUTATHIONE S-TRANSFERASE TAU 22              | 0.12          | 2.72         | 0.04              |            |
| AT5G49450 | BZIP1, BASIC LEUCINE-ZIPPER 1                        | 0.22          | 6.01         | 0.04              |            |
| AT5G47990 | CYP70S5A, CYTOCHROME P450, FAMILY 70S, SUBFAMILY A, POLYPEPTIDE 5 | 0.07          | 2.05         | 0.04              |            |
| AT1G02850 | BGLU11, BETA GLUCOSIDASE 11                           | 0.30          | 9.09         | 0.03              |            |
| AT2G17500 | PIL55, PIN-LIKES 5                                   | 0.08          | 2.45         | 0.03              |            |
| AT5G13080 | WRKY, WRKY DNA-BINDING PROTEIN 75                     | 0.20          | 6.49         | 0.03              |            |
| AT2G41380 | S-adenosyl-L-methionine-dependent methyltransferases superfamily protein | 0.06          | 2.09         | 0.03              |            |
| AT3G09270 | GSTU8, GLUTATHIONE S-TRANSFERASE TAU 8                | 0.13          | 5.50         | 0.02              |            |
| AT1G61560 | MLO6, MILDEW RESISTANCE LOCUS O 6                     | 0.18          | 7.34         | 0.02              |            |
| AT1G21100 | IGMT1, INDOLE GLUCOSINOLATE O-METHYLTRANSFERASE 1     | 0.09          | 3.96         | 0.02              |            |
EI11 and the expansin EXP17 showed a large high pH /low pH ratio, possibly to compensate compromised cell expansion at alkaline pH. This supposition is supported by a large high pH/low pH ratio of the auxin-related genes SAUR41 and SAUR72, which have been associated with the regulation of cell expansion [13, 18]. High pH/low pH ratios below one were observed for STRESS INDUCED FACTOR1 (SIF1), a root-specific, membrane-anchored LRR kinase with undefined function [33], the aluminum-activated malate transporter ALMT1, and the Cys2-His2 zinc-finger domain transcription factor SENSITIVE TO PROTON RHIZOTOXICITY 2 (STOP2). The latter two genes are inducible by low pH stress [14, 12]; almost complete repression was observed under the present (high pH) conditions. Notably, both genes are also induced by phosphate starvation, where ALMT1 and the STOP2 paralog STOP1 repress primary root growth in adaptation to decreased phosphate supply [1, 8]. Also, the auxin efflux carrier family proteins PILS3 and PILS5 [2] were strongly down- and highly upregulated by high and low pH, respectively, suggesting that ambient pH alters auxin homeostasis. A low high pH/low pH ratio was also observed for POLYGALACTURONASE INHIBITING PROTEIN 1 (PGIP1). PGIP1 stabilizes the cell wall under acidic conditions and was found to be dependent on STOP1/STOP2 [12]. Notably, PGIP1, STOP2, ALMT1, SIF1, and the S-adenosyl-L-methionine-dependent methyltransferases superfamily protein At2g4380 are regulated by STOP1 [12].

A subset of 12 genes was responsive to all three conditions under investigation and thus represents genes that are highly responsive to both changes in ambient pH and iron supply (Fig. 3b, c). This subset comprises CYP82C4 and various regulators such as SIF1, STOP2, and the FIT-regulated F-box/RNI superfamily protein At1g73120. Notably, CYP82C4, which was highly upregulated and downregulated under iron deficiency at optimal and high pH, respectively, was also upregulated at low pH conditions even when iron is sufficient, suggesting that the expression of CYP82C4 is dictated by external pH independent of the iron status.

**Discussion**

Alkaline soils are thought to aggravate iron deficiency by rendering the acquisition of iron pools more difficult due to decreased iron availability. Our data show that a difference in ambient pH from 5.5 to 7.0 is causative for considerable differentiation of the global gene expression profile, which goes well beyond simple exacerbation of the iron deficiency response. A relatively large set of genes is anti-directionally regulated by high and low pH, indicating that ambient pH is translated into transcriptional changes that adapt the plant to the prevailing hydrogen activity, a response that appears to be at least partly elicited by pH per se and independent of alterations in the availability of essential minerals such as phosphate and nitrate or toxic elements such as aluminum. Moreover, the iron deficiency response as such is modulated by alterations in ambient pH. While FRO2 expression is diminished at circumneutral pH, the production and secretion of iron-mobilizing coumarins is induced by high pH, prioritizing the most effective strategy to mobilize iron from otherwise inaccessible pools.

The transcriptional response of iron-deficient plants grown at high pH also revealed a pronounced overrepresentation of several categories containing genes that orchestrate the defense responses to pathogens, including

| Locus     | Description                                                                 | High pH (1 h) | Low pH (8 h) | High/low pH ratio |
|-----------|------------------------------------------------------------------------------|---------------|--------------|------------------|
| AT4G22610 | Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein | 0.11          | 5.37         | 0.02             |
| AT5G48010 | ATTHAS1, THALIANOL SYNTHASE, THALIANOL SYNTHASE 1                             | 0.05          | 3.00         | 0.02             |
| AT2G15490 | UGT73B4, UDP-GLYCOSYLTRANSFERASE 73B4                                        | 0.05          | 3.06         | 0.02             |
| AT5G06860 | PGIP1, POLYGALACTURONASE INHIBITING PROTEIN 1                                 | 0.06          | 4.10         | 0.01             |
| AT3G13950 | Ankyrin                                                                      | 0.06          | 7.14         | 0.01             |
| AT1G17170 | ATGSTU24, GLUTATHIONE S-TRANSFERASE TAU 24                                   | 0.13          | 18.19        | 0.01             |
| AT5G22890 | STOP2, SENSITIVE TO PROTON RHIZOTOXICITY 2                                   | 0.01          | 2.09         | 0.01             |
| AT1G05680 | UGT74E2, uridine diphosphate glycosyltransferase 74E2                         | 0.01          | 3.45         | 0.00             |
| AT1G51840 | SIF1, STRESS INDUCED FACTOR 1                                                | 0.03          | 10.81        | 0.00             |
| AT1G08430 | ALMT1, ALUMINUM-ACTIVATED MALATE TRANSPORTER 1                               | 0.03          | 15.97        | 0.00             |
| AT1G51830 | SIF1, STRESS INDUCED FACTOR 1                                                | 0.02          | 9.67         | 0.00             |
| AT4G31940 | CYP82C4, CYTOCHROME P450, FAMILY 82, SUBFAMILY C, POLYPEPTIDE 4               | 0.00          | 2.90         | 0.00             |
ERF- and WRKY-type transcription factors, PR proteins, and genes involved in proteolysis, secondary metabolism, and hormone signaling (Supplemental Figure 1). All these responses are much less pronounced in iron-deficient plants grown at optimal pH. Exposure to low pH with sufficient iron supply, on the other hand, elicited a more pronounced pathogen response, indicating pH-dependent prioritization of the responses to pathogen attack and iron starvation. In particular, genes related to signaling were overrepresented under low pH conditions. This survey shows that ambient pH considerably modulates the responses to environmental cues by altering the transcriptional landscape of iron-deficient plants to secure and optimize fitness of the plant under a given set of environmental conditions.

Conclusions
Our transcriptomic survey confirms previous observations that relatively small changes in media pH can have large impact on gene expression profiles. In addition, we show here that external pH significantly alters the response to iron deficiency by prioritizing specific, pH-dependent modules, allowing for a more efficient iron acquisition which is accurately tuned to a given set of edaphic conditions. Furthermore, the current data shed light on the intertwining of various signaling pathways, where external pH massively influence the decision-making process of plants by modulating the hierarchy of the responses to environmental stimuli.

Methods
Seeds of the Arabidopsis (Arabidopsis thaliana) accession Col-0 were obtained from the Arabidopsis Biological Resource Center (Ohio State University). Plants were grown under sterile conditions in a growth chamber on agar-based media as described by Tsai et al. [30].

The growth medium comprised 5 mM KNO$_3$, 2 mM H$_3$BO$_3$, 14 mM MnCl$_2$, 1 μM ZnSO$_4$, 0.5 μM CuSO$_4$, 0.01 μM CoCl$_2$, and 0.2 μM Na$_2$MoO$_4$, supplemented with 1.5% (v/v) sucrose, and solidified with 0.4% Gelrite pure (Kelco). For iron-deficient optimal-pH media, 180 μM FeCl$_3$ and 1 g/L MOPS were added, and the pH was adjusted to 5.5 with KOH. For iron-deficient high-pH media, 40 μM FeCl$_3$ and 1 g/L MOPS were added, and the pH was adjusted to 7.0 with KOH. Plants were grown on media for 14 d.

For RNA-seq analysis, total RNA was isolated from roots of 14-d-old plants grown under iron-deficient conditions either at optimal (5.5) or high (7.0) pH using the RNeasy Plant Mini Kit (Qiagen). Libraries for RNA-seq were prepared by using the Illumina TruSeq RNA library Prep Kit (RS-122-2001, Illumina) according to the manufacturer’s protocol. Briefly, 4 μg of total RNA per sample were used for library construction. PolyA RNA was captured by oligodT beads and fragmented when eluted. cDNA was synthesized from fragmented RNA using reverse transcriptase (SuperScript III, Cat. No. 18080–093, Invitrogen) and random primers. Reactions were cleaned up with Agencourt Ampure XP beads (Beckman Coulter Genomics). Libraries were end-repaired, adenylated at the 3’ end, ligated with adapters and amplified according to the TruSeq™ RNA Sample Preparation v2 LS protocol. Finally, the products were purified and enriched with 10 cycles of PCR to create the final double-stranded cDNA library. Final libraries were analyzed using Agilent High Sensitivity DNA analysis chip (Cat. no.5067–4626, Agilent) to estimate the quantity and size distribution, and were then quantified by qPCR using the KAPA Library Quantification Kit (KK4824, KAPA). The prepared library was pooled for single-end sequencing using Illumina HiSeq 2500 with 101-bp single-ended sequence read. Transcript abundance was calculated by first mapping reads to the Arabidopsis TAIR10 genome using Bowtie2 [15]. Unmappable reads were mapped to the TAIR 10 genome sequence by BLAT [9]. Read counts were computed using the RackJ package (http://rackj.sourceforge.net/) and normalized using the TMM-quantile method [20]. Normalized read counts were transformed into normalized RPKM values. Z-test was carried out for detecting differentially expressed genes. Two independent biological replicates were performed. RNA-seq data are available at BioProject ID PRJNA664641 (bioprojecthelp@ncbi.nlm.nih.gov).

Gene ontology (GO) enrichment analysis was analyzed using the Singule Enrichment Analysis (SEA) available on the ArgiGO v2.0 toolkit [29]. The analysis was performed using the following parameters: selected species: Arabidopsis thaliana; Reference: TAIR genome locus (TAIR10_2017); Statistical test method: Fisher; Multi-test adjustment method: Yekutieli (FDR under dependency); Significance level: 0.05; Minimum number of mapping entries: 5; Gene ontology type: Complete GO. Significantly enriched GO terms were summarized and visualized using REVIGO [26], with a similarity setting of 0.7 and SimRel as the semantic similarity measure. Final figures were plotted in R (version 3.6.2).

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s12864-020-07116-6.

Additional file 1: Supplemental Table 1. High-pH DEGs.
Additional file 2: Supplemental Table 2. Common DEGs between high-pH and optimal-pH datasets.
Abbreviations
RNA-seq: RNA-sequencing; GO: Gene ontology; qPCR: Quantitative polymerase chain reaction; RPKM: Reads per kilobase of transcript per million reads mapped

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Authors’ contributions
HJH contributed to the design of the study, carried out experiments, interpreted data and contributed to the manuscript. W.S. conceived and designed the study, participated in the analysis and interpretation of the data and wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The RNA-seq datasets are available at NCBI Sequence Read Archive under the accession number PRJNA664641.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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