1 Short title: Protein *S*-cyanylation in root hair development.

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5 **New insights on the role of HCN in root hair elongation through single cell**
6 **proteomics.**

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15

16 One sentence summary: Arabidopsis root hair proteomics unveils that several proteins
17 involved in root hair development are susceptible of modification by *S*-cyanylation.

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19

20 **Footnotes.**

21

22 List of author contributions: L.A-A. performed the experiments and analyzed the data;
23 M.Y supervised the experiments and analyzed the data; L.C.R analyzed the proteomic
24 data; L.C.R and I.G. conceived the project and wrote the article; I.G. supervised the
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42 proteomics, S-cyanylation, Arabidopsis.

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44

45 **Abstract.**

46

47 Root hairs are specialized structures involved in water and nutrient uptake by plants.
 48 They elongate from epidermal cells following a complex developmental program. β -
 49 cyanoalanine synthase (CAS), which is mainly involved in hydrogen cyanide (HCN)
 50 detoxification in *Arabidopsis thaliana*, plays a role in root hair elongation, as evidenced
 51 by the fact that *cas-c1* mutants show a severe defect in root hair shape. In addition to
 52 root hairs, CAS C1 is expressed in the quiescent center and meristem. However, the
 53 *cas-c1* mutation has no visible effect on either tissue, in both control and nutrient-
 54 deprivation conditions. To identify its role in root hair formation, we conducted single
 55 cell proteomics analysis by isolating root hair cells using Fluorescence-Activated Cell
 56 Sorting (FACS) from wild type and *cas-c1* mutants. We also analyzed the presence of
 57 S-cyanylation, a protein post-translational modification (PTM) mediated by HCN and
 58 affecting cysteine residues and protein activity, in proteins of wild type and *cas-c1*
 59 mutants. We found that several proteins involved in root hair development, related to
 60 the receptor kinase FERONIA signaling and to DNA methylation, are modified by this
 61 new post-translational modification.

62

Introduction

Root formation is one of the most important evolutionary events in the adaptation of plants to terrestrial ecosystems. Root hairs are specialized tubular structures of epidermal cells that improve the incorporation of nutrients and anchoring of the plant to the soil, as well as playing an important role in the interaction with other organisms (Cui et al., 2018).

In *Arabidopsis*, epidermal cells present a specific distribution that determines their fate: those that are between two cortical cells will become root hairs (H cells), and those in contact with the membrane of a single cortical cell will develop into non-hair (N) cells (Datta et al., 2011). The differentiation of N or H cells is determined by a plethora of regulatory proteins, which have been extensively studied (Grierson et al., 2014). Once cell fate is determined, H cells change their shape by modifying the cell wall to allow for root hair elongation. To accomplish this, ROP (“RHO-RELATED GTPase”) proteins are recruited by the FERONIA (FER) receptor kinase (Duan et al., 2010) and are concentrated in the root hair growth zone (Molendijk et al., 2001). At the same time, endoplasmic reticulum and filamentous actin are concentrated in this area. In addition, the pH decreases to 4-4.5, activating expansins and allowing the relaxation of the cell wall and the formation of the initial bulge (Monshausen et al., 2007). Elongation of the root hair is directed by vesicles produced by the rough endoplasmic reticulum and the Golgi complex, which release polysaccharides and cell wall glycoproteins, as well as protein synthases and membrane transporters through exocytosis at the tip of the root hair (Ketelaar et al., 2008). The control of membrane trafficking, therefore, is essential in the development of root hairs. RAB GTPases such as RAB-A4b (Rab GTPase Homolog A4b) play an important role in the regulation of vesicular traffic (Nielsen et al., 2008) by controlling the phosphorylation state of phosphatidyl inositol (PI) -PI4P (Thole et al., 2008) (Bubb et al., 1998; Yoo et al., 2012). Another ROP GTPase, ROP2 (Rho-related protein from plants 2) initiates ROS production by NADPH oxidase RHD2/ATRBOHD (Root hair defective 2) (Foreman et al., 2003; Jones et al., 2007). ROS promotes the entry of Ca^{2+} , which activates RHD2 in a positive feedback loop (Mendrinna and Persson, 2015). ROS production is also induced by the RSL4 protein (Root hair defective 6 like 4), which controls the expression of NADPH oxidases and peroxidases. These also participate in the production of ROS in root hairs (Hwang et al., 2017). The Ca^{2+} gradient at the tip of the root hair directs the growth, facilitates the

97 fusion of the vesicles with the apical plasma membrane and contributes to the release of
98 the vesicular content necessary for the expansion of the cell wall (Pei et al., 2012). The
99 organization of the cytoskeleton, hormones such as ethylene and auxin as well as
100 nutrient availability are also essential in the formation and development of root hairs
101 (Ketelaar, 2013) (Sieberer et al., 2005). (Jones et al., 2009) (Cui et al., 2018) (Jungk,
102 2001).

103 Hydrogen cyanide (HCN) is synthesized in plants as a co-product during ethylene
104 biosynthesis but also is produced by several plant growth-promoting rhizobacteria. It
105 acts as a biocontrol agent and influences nutrient availability (Peiser et al., 1984;
106 Rijavec and Lapanje, 2016). The role of HCN as a signaling gasotransmitter involved in
107 root hair development and the plant immune response has been widely described.
108 Recently, it has also been described as a signaling molecule in mammalian systems
109 (Garcia et al., 2010; Garcia et al., 2013; Gotor et al., 2019; Pacher, 2021; Zuhra and
110 Szabo, 2021). HCN is mainly detoxified by the β -cyanoalanine synthase CAS-C1.
111 Maintaining low levels of HCN by CAS-C1 is required for proper root hair
112 development (Garcia et al., 2010). CAS-C1 accumulates in root hair tips during root
113 hair elongation. Thus, higher accumulation of HCN in *cas-c1* inhibits the action of the
114 NADPH oxidase gene *ROOT HAIR DEFECTIVE 2 (RHD2)/AtrbohC* in a manner
115 independent of enzyme inactivation (Arenas-Alfonseca et al., 2018b, 2018a). Root hairs
116 of *cas-c1* are specified correctly, but they do not elongate. Genetic analysis showed that
117 the mutant of the *SUPERCENTIPEDE (SCN1)* gene is epistatic to *cas-c1* and *rhd2*
118 (Arenas-Alfonseca et al., 2018b, 2018a). HCN acts as a signaling molecule that
119 influences root hair development in a manner independent of ROS formation and of
120 direct NADPH oxidase inhibition, and its action has been localized in the first steps of
121 root hair elongation, between SCN1 action and RHD2-driven production of $O_2^{\cdot-}$
122 (Arenas-Alfonseca et al., 2018b, 2018a).

123 The mechanisms that underlie HCN modulation, the mode of action and the specific
124 targets of this molecule are areas of active research. HCN is able to produce post-
125 translational modifications in the thiol groups of cysteines, producing S-cyanocysteine.
126 Our research has shown that some *Arabidopsis* proteins are naturally S-cyanylated and
127 that this modification can change the activity of these proteins (Garcia et al., 2019)
128 suggesting that the S-cyanylation represents a new mechanism of regulation of the
129 processes in which HCN plays a signaling role.

130 Although CAS-C1 is expressed in the meristem in addition to root hairs, we show that
 131 *cas-c1* does not affect meristem formation or root hair cell fate. In order to identify the
 132 protein targets of cyanide in the root hair, we carried out a cell-type specific proteomic
 133 approach to characterize the proteins present in wild-type root hair cells and compare
 134 them to those present in *cas-c1* mutant cells. Through this analysis, we found that
 135 several proteins involved in root hair development are modified by *S*-cyanylation.
 136
 137

138 **Results**

139

140 ***CAS-C1* transcription is cell-type specific**

141

142 A cell-type specific transcriptomic analysis (Li et al., 2016b), showed that *CAS-C1* is
 143 highly expressed in several root cell types, such as the quiescent center (QC, *WOX5*-
 144 tagged cells), the phloem pole pericycle (S17-tagged cells), developing cortex (*CO2*-
 145 tagged cells) and mature cortex (*COR*-tagged cells) (Supplemental Fig. S1A). Also,
 146 *CAS-C1* transcription increased in the elongation zone compared to the meristematic
 147 zone, and remained high in the differentiation zone (Supplemental Fig. S1B). These
 148 results suggested a possible developmental role of *CAS-C1* in the root of *Arabidopsis*
 149 *thaliana*.

150

151 ***CAS-C1* is not involved in QC formation, meristem length or root elongation**

152

153 To determine if *cas-c1* mutants are affected in QC establishment or maintenance, we
 154 carried out confocal microscopy of wild type and *cas-c1* mutant meristems stained with
 155 propidium iodide to visualize cell walls. Fig. 1 shows that no differences were found
 156 either in the number or morphology of QC cells in roots grown in control conditions.
 157 No differences were also observed in meristem size of wild type and *cas-c1* (white
 158 arrows in Fig. 2A mark the transition zone between meristematic and elongation zones).
 159 Time-lapse measurements of root length (Fig. 2 and Supplemental Fig. S2) confirmed
 160 that root elongation was identical in both genotypes.

161 The *CAS-C1* protein is involved in mitochondria sulfur metabolism. It detoxifies
 162 hydrogen cyanide by using as a substrate the sulfur-containing amino acid cysteine
 163 (Romero et al., 2014). It is also indirectly involved in nitrogen recycling because the
 164 nitrogen residue in HCN can be incorporated into amino acids through the nitrilase
 165 activities of β -cyanoalanine, the product of HCN detoxification by *CAS-C1*
 166 (Piotrowski, 2008). To determine if root growth is affected by loss of these metabolic
 167 functions, the root phenotype was analyzed in conditions of nitrogen and sulfur
 168 deficiency. Wild type and *cas-c1* mutant plants were grown on vertical plates of MS
 169 medium and different nutrient-deprived media for 9 days and root length was measured
 170 (Fig. 3 and Supplemental Fig. S3). No differences were observed in any condition
 171 between wild type and *cas-c1* (Supplemental Fig. S4). Phosphate starvation induces,

Figure 1

Fig. 1. Root meristem phenotype in wild type and *cas-c1* mutant. Col-0 (A) and *cas-c1* mutant (B) seedlings were grown for 7 d on MS medium supplemented with sucrose in vertical plates. Representative images are shown. White squares indicate QC cells. Propidium iodide was used to visualize cell walls.

among other root phenotypes, root hair elongation, involving auxin, ethylene, cytokinin and post-translational modifications (Huang and Zhang, 2020). Therefore, as *cas-c1* mutants show a defect in root hair formation, we included phosphate starvation in our observations. Again, no differences were found in Pi starvation (Fig. 3 and Supplemental Figs. S3C and S4D). Taken together our results indicate that *cas-c1* does not affect root growth in control or under N, S or P nutritional stress conditions.

178

179 **CAS-C1 is not involved in root hair cell fate**

Because *cas-c1* has a severe defect in root hair morphogenesis (Garcia et al., 2010; Arenas-Alfonseca et al., 2018a, 2018b), we hypothesized that it may also have altered root hair cell fate. To visualize root hairs during development, we crossed into *cas-c1* the COBRA-like protein 9 (COBL9) promoter driving GFP, which is root hair specific (Brady et al., 2007b) (Petricka et al., 2012). The *cas-c1*; pCOBL9:GFP line showed a normal patterning of green fluorescence (Fig. 4), both in its abnormal root hairs and in its root hair primordia, indicating that the patterning of root hair formation is not altered in this mutant. Thus, *cas-c1* affects root hairs exclusively in their elongation.

188

189 **Proteomics of wild-type and *cas-c1* root hairs**

Figure 2

Fig. 2. Root meristem size in MS medium. A) A representative root of 7 days-old wild type (wt) and *cas-c1* mutant was stained with propidium iodide. White arrows indicate the transition between the elongation and transition zones in wild type and *cas-c1* mutant seedlings. Representative images are shown. B) Root meristem length of wild type and *cas-c1* mutants in MS medium. Media $\pm$ SD are shown in the graph. N>20.

190 To analyze the effect of the *cas-c1* mutation, we isolated 10^6 root hair cells by
 191 Fluorescence-activated cell sorting (FACS) from three independent replicates of WT
 192 pCOBL9:GFP and *cas-c1* pCOBL9:GFP roots. The extracted proteins from each
 193 sample were trypsin-digested and the peptides analyzed by liquid chromatography-high
 194 resolution mass spectrometry (LC-MS/MS).

195 In wild-type samples, we identified 3,829 unique proteins at a false discovery rate
 196 below 1% (FDR<1%), which represent almost 10% of the total *Arabidopsis* proteome
 197 (Dataset S1). Previous work identified 1,387 proteins in root hair cells by a similar
 198 experimental approach using GeLC-MS/MS analysis (Petricka et al., 2012) (Fig. 5A),
 199 90% of which we also identified. In protein extracts of the *cas-c1* pCOBL9:GFP roots,
 200 we identified 3,972 proteins (Dataset S2) of which 3,515 were common in both cell
 201 types, 310 were only identified in wild type and 457 only in *cas-c1* (Fig. 5B).

202

203 *cas-c1* root hair cells differ from non-hair cells at the proteomic level

204 To understand the stage at which *cas-c1* root hair development is blocked, we compared
 205 proteins present in *cas-c1* and in wild-type root hairs. To this end, we compared *cas-c1*
 206 and wild-type RH cells with published proteomics data of hair/non hair (NH) epidermal
 207 cells (Petricka et al., 2012). Of the 457 proteins present only in *cas-c1* RH and the 113

Figure 3

Fig 3. Root length measurement in nutrient deficient media. Wild type and *cas-c1* mutant seedling were grown for 9 d in vertical plates of MS medium or MS medium in the absence of different nutrient compounds. Root length was measured with ImageJ software. Values are means $\pm$ SD of three independent experiments. **, $P < 0.01$

208 specific to NH cells, only 5 proteins were coincident (Supplemental Fig. S5). Thus, at
 209 the protein level, *cas-c1* RH are not equivalent to NH cells. This is consistent with the
 210 *cas-c1* phenotype, which has root hairs, although they are short and unstructured. A
 211 similar result was obtained by comparing the proteome of *cas-c1* RH with the wild-type
 212 NH proteome in (Lan et al., 2013), in which another RH-specific promoter, EXP7, was
 213 fused to GFP and root hair cells isolated by FACS. Thus, these proteomic comparisons
 214 show that *cas-c1* root hairs are not similar to non-hair cells and they have their own
 215 identity. A deeper analysis of the root hair proteome was therefore undertaken.

216

217 ***feronia* and *cas-c1* mutants share common features**

Figure 4

Fig. 4. GFP localization in roots from the wt-pCOBL9:GFP and *cas-c1*-pCOBL9:GFP transgenic lines. Plants were grown 6 days in MS medium. Representative images are shown.

Several *Arabidopsis* mutants have a phenotype similar to *cas-c1* including the basic helix-loop-helix (bHLH) transcription factor ROOT HAIR DEFECTIVE 6-LIKE 4 (*rsl4*), which is a target of the bHLH transcription factor ROOT HAIR DEFECTIVE 6 (*rhd6*) and acts as signal to induce cell growth in root hairs (Menand et al., 2007; Yi et al., 2010). We thus compared the transcriptomic data from the genes mis-regulated in *rsl4* (Yi et al., 2010) with the proteins present only in wild-type or in *cas-c1* root hairs. The comparison showed that the number of common genes/proteins was very low and therefore we cannot establish a relationship between these mutations (Supplementary Fig. S6).

RHO GTPases are a large family of related monomeric GTP-binding proteins that serve diverse signaling functions (Etienne-Manneville and Hall, 2002). Plant ROPs are a unique clade of RHO-like GTPases involved in cell growth and polarity establishment among other functions (Yang and Fu, 2007; Kost, 2008). FERONIA is a ROP-interacting receptor-like kinase (RLK) and regulates ROP-signaling and ROS-mediated root hair development, as well as many other aspects of plant growth and development (Hématy and Höfte, 2008). We found that *feronia* (*fer*) mutants show a high percentage of up or down-regulated genes common to the proteins that are present in *cas-c1* root

Figure 5

Fig. 5. A, Venn diagram showing the unique and intersection of the identified protein in the proteomic analysis of Petricka et al, 2012 and this work . B, Venn diagram showing the unique and intersection of the identified proteins in wild type and *cas-c1* root hair samples.

235 hairs (Supplemental Fig. S7), as well as a very similar phenotype to *cas-c1* at the root
 236 hair level (Duan et al., 2010). This suggested a functional relationship between *fer* and
 237 *cas-c1* mutations.

238

239 Functional classifications of root hair-specific proteins

240 To identify the function of CAS-C1 and explain the hairless phenotype of the mutant we
 241 addressed the functional classification of the proteins identified in wild-type and *cas-c1*
 242 root hair samples based on the information available in three gene/protein databases:
 243 Uniprot for Gene Ontology classification (Bairoch et al., 2005; Pundir et al., 2016),
 244 MapMan nomenclature developed for plant-specific pathways and processes (Navarro
 245 et al., 2019), and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database for
 246 identification of overrepresented pathways (Kanehisa et al., 2016). Gene Ontology (GO)
 247 classification by biological processes of the identified proteins indicated that most of the
 248 proteins are involved in “Cellular (GO:0009987) and Metabolic (GO:0008152)
 249 processes.” This classification might be biased by the fact that these processes include
 250 the most abundant proteins, which are preferentially detected by MS, as observed in
 251 other proteomic analyses (Aroca et al., 2017; Huang et al., 2019) (Supplemental Fig.
 252 S8). An important subset of proteins is categorized within the GO “Response to
 253 stimulus” (GO:0050896), which includes ten secondary GO identifications, highlighting

254 “Response to stress” and “Response to chemical” (Supplemental Fig. S8). GO
 255 classification of the proteins present only in wild type or *cas-c1* root hair samples, again
 256 showed that “Cellular and Metabolic processes” are the prevailing sets but within the
 257 GO classification “Biological regulation” (GO:0065007) there were 66 and 136 proteins
 258 in wild type and *cas-c1*, respectively (Supplemental Fig. S9). Among the proteins in this
 259 GO subclassification (Supplemental Table S1), we identified the transcription factor
 260 ELONGATED HYPOCOTYL 5 (HY5), a positive regulator of photomorphogenesis
 261 whose absence results in increased root hair length (Zhang et al., 2019),
 262 TRANSPARENT TESTA GLABRA 1 (TTG1), involved in trichome and root hair
 263 development (Tan et al., 2021), and ENHANCED ETHYLENE RESPONSE PROTEIN
 264 5 (EER5), which functions in resetting the ethylene-signaling pathway (Christians et al.,
 265 2008). In this subset of proteins, we also identified in *cas-c1* but not in wild type,
 266 several proteins involved in hormone signal transduction such as the brassinosteroid co-
 267 receptor BRI1-ASSOCIATED RECEPTOR KINASE 1 (BAK1) and the
 268 BRASSINOSTEROID-SIGNALING KINASE 7 (BSK7), and auxin signaling and
 269 trafficking such as the AUXIN SIGNALING F-BOX2 (AFB2) and SHORT AND
 270 SWOLLEN ROOT 1 (NDP1/SSR1), which encodes a tetratricopeptide-containing repeat
 271 localized in mitochondria involved in root development (Han et al., 2021).

272 The identified proteins in both sample types were also analyzed based on their assigned
 273 functions and classified into 35 functional groups using the MapMan nomenclature
 274 (Navarro et al., 2019) (Fig. 6). The most abundant set corresponded to the general
 275 PROTEIN group, which included almost 20% of the total identified proteins in both cell
 276 type backgrounds with 853 and 898 elements in wild type and *cas-c1*, respectively. This
 277 bin set comprises several subsets involved in protein activation, protein synthesis and
 278 degradation, protein targeting, post-translational modification, protein folding and
 279 glycosylation (Supplemental Table S2). Taken into account the defect in development
 280 of the root hairs in *cas-c1*, we analyzed in detail the proteins involved in developmental
 281 processes in the protein subset present only in the samples of wild type and *cas-c1*. As
 282 shown in Supplemental Table S3, 7 and 16 proteins classified in development are only
 283 present in wild type and *cas-c1*, respectively. Among the proteins that are absent in *cas-*
 284 *c1* root hair-like cells, we found the protein kinase TARGET OF RAPAMYCIN (TOR),
 285 which functions as a central regulator of growth, coordinating nutritional and hormonal
 286 signaling, as well as a repressor of the autophagy process (Xiong and Sheen, 2014),
 287 EMBRYO DEFECTIVE 30 (EMB30), a GDP/GTP exchange factor for small G-

Figure 6

Fig. 6. Bubble plot of the functional classification of the identified proteins according to the plant-specific database MapMan. Bubble columns represent the proteins classified within each bin of the total proteins identified in wild type (wt), *cas-c1* root hair samples, and those only present in wt or *cas-c1* root hair samples. Numbers besides the bubble represent the amount of proteins classified within the bin.

proteins involved in the specification of apical-basal pattern formation and essential for cell division and expansion (Li et al., 2016a), and HSP4/SABRE protein, which mediates microtubule organization and affects root hair patterning (Pietra et al., 2015). Among the proteins that are only present in *cas-c1*, we identified PESCADILLO, which plays a role in root elongation and differentiation (Zografidis et al., 2014), and TRANSPARENT TESTA GLABRA 1 (TTG1), also identified in the GO classification of Biological regulation.

Finally, the proteins that were present only in wild type or *cas-c1* samples were also analyzed using the KEGG Search Pathway (Supplemental Table S4) to identify overrepresented pathways or processes. Apart from the Metabolic and Secondary metabolite biosynthetic pathways, the Spliceosome pathway is the one that shows the highest representation of proteins in *cas-c1* root hair samples with 22 proteins (Supplemental Table S5 and Supplemental Fig. S10). These 22 proteins include components of the small nuclear ribonucleoproteins U1, U2, U4, U5, U6 complex like SPF3 (At2g02570) and YLS8 (At5g08290); the Prp19 complex, and the Prp2 and Prp22 pre-mRNA-splicing factors RNA helicase. This is not surprising because the spliceosome is modified in root hair cells as compared to non-hair cells, and there is less

intron retention in RH than in NH cells (Lan et al., 2013). In addition, the RNA transport and mRNA surveillance pathway are also overrepresented with 12 and 9 proteins respectively.

***S*-cyanylation of root hair proteins**

HCN can produce post-translational modification of proteins by *S*-cyanylation of cysteine residues (Garcia et al., 2019). *S*-cyanylation in peptides can be identified by a 25.0095 Da mass increase in the fragmentation spectrum. We identified 25 proteins *S*-cyanylated in wild type (Table 1) and 32 in *cas-c1* plants (Table 2), 8 of which are common in both. The low number of modified proteins identified is biased by the fact that a chemical selective method to label, enrich and detect *S*-cyanylated proteins is not available even though such does exist for other post-translational modifications including phosphorylation (Dudley and Bond, 2014), nitrosylation (Qu et al., 2016) or persulfidation (Aroca et al, 2017). Among the *S*-cyanylated proteins identified in *cas-c1* root hair samples, we observed four proteins of the methionine regeneration SAM cycle including COBALAMIN-INDEPENDENT METHIONINE SYNTHASE (MS1), METHIONINE SYNTHASE 2 (MS2), the *S*-ADENOSYL-L-HOMOCYSTEINE (SAH) HYDROLASE 2 (SAHH2) and the *S*-ADENOSYL-L-HOMOCYSTEIN HYDROLASE 1 (SAHH1/HOG1). These proteins have been previously detected as *S*-cyanylated in *cas-c1*, which indicates that the SAM cycle is a significant target process of HCN (Garcia et al., 2019). Fig. 7 shows the digestion pattern of the peptide containing the *S*-cyanylated Cys in SAHH1/HOG1. As shown in the table, a predicted ion containing Cys-CN is identified in the spectrum. Interestingly, *hog1* mutants show a root hair defective phenotype, similar to that of *cas-c1* (Wu et al., 2009).

We also found that the GLYCINE-RICH RNA BINDING PROTEIN 7 (GRP7) is *S*-cyanylated in the two sets of proteins. Fig. 8 shows the digestion pattern of the peptide containing the *S*-cyanylated Cys in GRP7 and predicted ions containing Cys-CN are identified in the spectrum. GRP7 binds FER and is phosphorylated in the RAPID ALKALINIZATION FACTOR PEPTIDES (RALF)-FER-GRP7 complex, which is implicated in RNA alternative splicing during root hair formation (Wang et al.). This, together with the *in silico* data obtained in a previous section, supports a relationship between *cas-c1*/HCN and *feronia*.

Fig. 7. LC-MS/MS mass spectra of the tryptic peptide containing the S-cyanlated Cys³⁴⁶ residue of the S-Adenosyl homocysteine hydrolase SAHH1/HOG1 protein. The table contains the predicted ion type for the modified peptide, and the ions detected in the spectrum are highlighted in green color.

Discussion

The role of ROS, particularly $O_2^{\bullet-}$ and H_2O_2 , in root and root hair development has been extensively studied. These species exhibit different gradient distributions in the roots (Yamada et al.; Zhou et al., 2020). A gradient of $O_2^{\bullet-}$ is essential for root hair elongation and this gradient at the root hair tip is lacking in the *cas-c1* mutant (Garcia et al., 2010). On the other hand, a transcriptomic analysis suggested that *CAS-C1* might play a role in QC, phloem or developing cortex formation or function (Lan et al., 2013). Despite the fact that *cas-c1* plants have a higher ROS content than wild type in control conditions (Garcia et al., 2013), no visible differences were detected in the QC, developing and maturation zone morphology or root length in *cas-c1* as compared to wild type. In the root maturation zone, the epidermal cells located in the H cell position emerge to form hair cells, and plant hormones, such as auxin and ethylene regulate root hair formation *via* ROS. Moreover, superoxide anion drives the elongation of the root hair *via* NADPH oxidase action (Carol and Dolan, 2006). However, the *cas-c1* phenotype is not related to ROS even though there is a possible direct inhibition of the NADPH oxidase RHD2/ATRBOHD by HCN (Arenas-Alfonseca et al., 2018a, 2018b). Thus, the proteomic analysis performed on isolated root hair cells that is described here provides additional functional data for the *CAS-C1* protein and HCN role in root hair development.

Fig. 8. LC-MS/MS mass spectra of the tryptic peptide containing the S-cyanlated Cys¹⁰ residue of the Glycine Rich Protein 7, (GRP7). The table contains the predicted ion type for the modified peptide, and the ions detected in the spectrum are highlighted in green color.

359

360 Previously, there has been little proteomic analysis performed on root hair cells

361 (Petricka et al., 2012) (Lan et al., 2013). In this work, more than 3700 proteins were

362 identified in each sample, of which 457 were identified in *cas-c1* but not in wild type.

363 This demonstrated that the suppression of CAS-C1 activity and the consequent

364 accumulation of HCN in the *cas-c1* mutant generates important changes in the root hair

365 proteome (Garcia et al., 2010; Arenas-Alfonseca et al., 2018a, 2018b). The analysis of

366 these 457 proteins using the KEGG database showed an over-representation of proteins

367 involved in the spliceosome, the RNA transport and mRNA monitoring pathways. The

368 spliceosome is a large complex formed by small nuclear ribonucleoproteins (snRNPs)

369 (Lorković et al., 2000) that process introns in pre-mRNA to convert it into mature

370 mRNA, a fundamental stage in gene expression in eukaryotes. RNA binding proteins,

371 RNA dependent ATPases, RNA helicases and protein kinases are also involved in this

372 process. Many of the spliceosome proteins, identified exclusively in *cas-c1*, participate

373 in various developmental processes, including flowering, apical dominance, leaf and

374 rosette size, shape and morphology (Lopato et al., 1999; Ru et al., 2008). An in-depth

375 study of gene expression in specific root cells showed great variation in the presence of

376 RNAs with non-coding intergenic regions that contribute to the functional specialization

377 of the cell, demonstrating that alternative intron processing serves to regulate

378 differentiation (Li et al., 2016b). Therefore, RH display less intron retention than NH

379 and this proteomic difference between *cas-c1* and wild type plants is consistent with this
380 finding.

381

382 It is noteworthy that we only detected TRANSPARENT TESTA GLABRA1 (TTG1) in
383 *cas-c1* root hairs. This protein, together with WEREWOLF (WER) and GLABRA3
384 (GL3), form a complex in non-hair cells that induces GLABRA2 (GL2) expression and
385 controls non-root hair cell fate by the GL2-mediated suppression of a set of root hair
386 specific transcription factors (Shibata and Sugimoto, 2019). Therefore, a *ttg1* knockout
387 mutant displays a hairy phenotype. The presence of abnormally high amounts of TTG1
388 in the *cas-c1* root hairs could result in a hairless phenotype. However, we demonstrate
389 that the *cas-c1* mutation does not affect root hair cell fate as a COBL9-GFP fusion
390 locates exclusively in hair cells and follows the wild-type alternate H-NH pattern, and
391 COBL9 action locates downstream of TTG1 in the genetic pathway that leads to root
392 hair specification and elongation (Grierson and Schiefelbein, 2009).

393

394 Previous genetic experiments demonstrated that CAS-C1 acts between
395 SUPERCETIPEDE 1 (SCN1), a Rho GTPase GDP dissociator inhibitor, which acts as a
396 negative regulator of ROPs, and the NADPH oxidase action, which is regulated by
397 ROPs (Arenas-Alfonseca et al., 2018b, 2018a). This points to ROPs as possible targets
398 of cyanide action. FERONIA regulates ROP-signaled root hair development (Duan et
399 al., 2010), and the transcriptional profile of its mutant shows a significant number of
400 mis-regulated genes common to mis-regulated proteins in *cas-c1*. Thus, we can
401 hypothesize that both *fer* and *cas-c1* share common elements in the root hair
402 development process or that one acts upstream of the other. This may involve S-
403 cyanylation of proteins regulated by FER in RH elongation as a mode of action of HCN.
404 Candidates could be peroxidases, ROPs or other proteins involved in RH elongation
405 mediated by FER. This opens an interesting focus on the interactions between *cas-c1*,
406 *feronia* and S-cyanylation that will be the subject of future research. On the other hand,
407 it has been demonstrated that the interaction between the peptide rapid alkalization
408 factor (RALF) and FERONIA modulates protein synthesis by the phosphorylation of a
409 translation initiation factor (Zhu et al., 2020). Several initiation factors have been
410 identified in samples of both wild-type and *cas-c1* root hairs. The translation initiation
411 factor 2 is present only in *cas-c1* root hairs, as well as another putative initiation factor,
412 thus establishing a possible relationship between FERONIA and CAS-1 at the

functional level. This is consistent with the MapMan analysis, in which approximately 20% of the genes belong to PROTEIN group, opening also the question of whether proteins are affected at the post-translational level by HCN and its associated post-translational modification, the S-cyanylation (Garcia et al., 2019). Moreover, GRP7 is S-cyanylated in root hairs, being a protein that works together with the RALF-FER module to adjust the alternative RNA splicing in response to environmental and developmental factors (Wang et al.). Therefore, HCN signaling and root hair elongation could be coupled by the S-cyanylation of this RNA-binding protein and its interaction with FERONIA.

Among the proteins that are S-cyanylated in *cas-cl* plants, we found a significant number of members of the SAM cycle, a relevant finding because in our previous work we also found these proteins in *cas-cl* samples (Garcia et al., 2019). The SAM cycle is involved in DNA methylation through SAM, a methylation DNA agent. DNA methylation is a conserved epigenetic marker that regulates many developmental and stress responses and adaptation in plants, including the transmission of a stress memory that can be important in the response to pathogens. Mutant lines (*sahh1/hog1*) with reduced levels of the S-ADENOSYL-L-HOMOCYSTEIN HYDROLASE 1 enzyme show a deficiency in root hair development pointing to a relationship between methylation level and RH development (Wu et al., 2009). There is substantial evidence that correlates the levels of SAM cycle components with DNA methylation changes (Zhang, 2018; Gonzalez and Vera, 2019). In plants, inhibition of the METHIONINE SYNTHASE interferes with the immune response of the plant to pathogens. It has been demonstrated extensively that *cas-cl* plants are more resistant to biotrophic pathogens (Garcia et al., 2013; Garcia et al., 2014; Arenas-Alfonseca et al., 2021). In fact, very low concentrations of KCN (1 μ M) are able to induce the plant response to pathogens and induce the *PATHOGENESIS-RELATED 1 (PRI)* gene (Arenas-Alfonseca et al., 2021), and it has been very recently described that this fact is not exclusive to plants. In mammalian cells, very low concentrations of HCN (from nM to 1 μ M) induce cellular proliferation and bioenergetics via cytochrome C oxidase stimulation (Randi et al., 2021). Understanding the participation of HCN in regulating the SAM cycle and DNA methylation in plant priming is one of our future goals.

Materials and methods

447

448 **Plant material and growth conditions**

449 *Arabidopsis* (*Arabidopsis thaliana*) wild type ecotype Col-0 and the *cas-cl* T-DNA
450 insertion mutant (*cas-cl*; SALK_103855; (Garcia et al., 2010)) were grown in soil or
451 vertical positioned plates in a photoperiod of 16 h of white light ($120 \mu\text{mol m}^{-2} \text{s}^{-1}$) at
452 20°C and 8 h of dark at 18°C. Seeds were surface sterilized using 50 % (vol/vol) bleach
453 and 0.1% Tween 20 (Sigma) for 15 min and then rinsed five times with sterile water.
454 All seeds were plated on standard MS media ($1\times$ Murashige and Skoog salt mixture,
455 Caisson Laboratories), 0.5 g/L MES, 1% Sucrose, and 1% Agar (Difco) and adjusted to
456 pH 5.7 with KOH. All plated seeds were stratified at 4 °C for 2 d before germination.
457 For nutrient deficiency assays, 1x Murashige and Skoog lacking sulphur, nitrogen or
458 phosphate (Caisson Laboratories) were used.

459

460 **Root meristem visualization**

461 For root meristem analysis, wild type and *cas-cl* mutant plants were grown for 7 days
462 in vertical MS medium plates. Roots were then mounted in 10 mg/mL propidium iodide
463 (PI) in water and observed using a 20 X objective with a Zeiss LSM 880 laser scanning
464 confocal microscope. Excitation and detection window were set as follows: excitation at
465 561 nm and detection at 570-650 nm.

466

467 **Expression of pCOBL9:GFP in roots**

468 The pCOBL9:GFP construct (Brady et al., 2007a; Petricka et al., 2012) was introduced
469 into wild type (Col) and *cas-cl* mutant plants. Two independent T3 lines in each
470 background were grown for 6 days in MS media. Roots from transgenic plants were
471 imaged using a Leica TCS SP2 spectral confocal microscope. Samples were excited
472 using an argon ion laser at 488 nm; emission was detected between 510 and 580 nm for
473 GFP imaging (pseudocolored green). The microscopy images were processed using
474 Leica Confocal Software.

475

476 **Root protoplasts isolation**

477 500-1000 wild type and *cas-cl* mutant seeds were grown for 6 days on Nitex nylon
478 mesh MS vertical plates (100mm x 100mm x15mm). Roots were sliced and placed in a
479 petri dish (Falcon 351008) holding one 70 μm strainer (Falcon 352350) with 7 ml buffer
480 B (cellulase 15 g l⁻¹, (Sigma #C1794) and pectolyase 1 g l⁻¹, (Sigma #P3026) in buffer

481 A). Roots were incubated for 1 h at room temperature and 85 rpm. The filtered liquid
482 was transferred to 15 ml Falcon and centrifuged for 6 min at 22 °C, 200 x g. Supernatant
483 was then removed and the pellet was resuspended in 300 µl of buffer A (mannitol 600
484 mM, MgCl₂ 2 mM, CaCl₂ 2 mM, MES 2 mM, KCl 10 mM and BSA 0,1% (p/v), pH
485 5.5). The mix was after filtered in a 70 and 40 µm (Falcon 352340) strainer,
486 respectively, and the filtered cells were kept in a 5 ml polystyrene tube.

488 **Fluorescent activated cell sorting (FACS)**

489 Protoplasts from wild type pCOBL9:GFP and *cas-c1* pCOBL9:GFP transgenic plants
490 were selected in a sorter MoFlo Astrios EQ (Beckman-Coulter), with a 70 µm nozzle at
491 a rate of 5,000 to 10,000 events per second and a fluid pressure of 60 psi. Protoplast of
492 non-transgenic wild type and *cas-c1* plants were used as negative control. GFP positive
493 cells were based on the negative control. They were read using a bandpass filter of
494 526/52. Protoplast were then collected in PBS buffer (NaCl 80 g l⁻¹, KCl 2 g l⁻¹,
495 Na₂HPO₄ 14.4 g l⁻¹ y KH₂PO₄ 2.4 g l⁻¹, pH 7.4), frozen in liquid nitrogen and stored for
496 protein extraction.

498 **LC-MS/MS based root hair proteomics**

499 Protein extraction from sorted protoplast was done and protein amount was measured
500 by Bradford assay (Bradford, 1976) and normalized with 50 mM NH₄HCO₃ pH 8.0 to
501 0.1-1 µg µl⁻¹. RapiGest SF Surfactant (Waters) 0.2% (v/v) was added. Proteins were
502 incubated 10 min at 40 °C, 100 rpm and reduced with DTT 10 mM. After, proteins were
503 incubated again at 10 °C for 40 min and 100 rpm. Samples were cool down at room
504 temperature and alkylated with iodoacetamide for 30 min at RT. Protein digestion was
505 done with trypsin (Promega) at a 1:50 ratio and incubated for 4 h. Peptides were dried in
506 a vacuum centrifuge. Prior to LC-MS/MS analysis, samples were resuspended in TFA
507 1 % (v/v), acetonitrile 2 % (v/v). Samples were then incubated for 2h at 60 °C and 100
508 rpm and centrifugated at 15000 rpm for 5 min. Supernatant was stored for LC-MS/MS.
509 1 µg of protein was analyzed in a LC-MS/MS elution gradient for 90 min in a mass
510 spectrometer “Q Exactive HF-X Hybrid Quadrupole-Orbitrap”, (Thermo Scientific).
511 LCMS and MS/MS data was process by Proteome Discoverer 2.2 program (Thermo
512 Scientific) and analyzed for identification in MASCOT (Matrix Science) against
513 TAIR10. Search tolerances were 5 ppm for precursor ions and 0.02 Da for product ions

514 using trypsin specificity with up to two missed cleavages. All searched spectra were
515 imported into Scaffold (v4.3, Proteome Software) and scoring thresholds were set to
516 achieve a peptide false discovery rate of 1% using the PeptideProphet algorithm.

517

518 **Proteomic analysis**

519 Protein function analysis and classification was performed using three biological
520 gene/protein databases: MapMan (Klie and Nikoloski, 2012), Uniprot (Bairoch et al.,
521 2005; Pundir et al., 2016) and the Kyoto Encyclopedia of Genes and Genomes (KEGG)
522 (Kanehisa et al., 2016).

523

524 **Accession Numbers**

525 Sequence data from this article can be found in the website <http://www.arabidopsis.org/>
526 for Arabidopsis genes with locus identifiers provided in this study. The mass
527 spectrometry proteomics data have been deposited in the ProteomeXchange Consortium
528 via the PRIDE partner repository with the data set identifier PXD028991 (Vizcaíno et
529 al.).

530

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532

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543

544

Table 1. Cyanilated proteins identified in wild type root hair samples

AGI	Gene alias	Annotation
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AT1G11910	APA1	APA1 aspartic proteinase A1
AT1G26630	ATELF5A-2FBR12	ATELF5A Eukaryotic translation initiation factor 5A-1 (eIF-5A 1) protein
AT3G52930	AtFBA8	AtFBA8 Aldolase superfamily protein
AT2G44100	ATGDI1	ATGDI1__guanosine nucleotide diphosphate dissociation inhibitor 1
AT2G21660	ATGRP7	ATGRP7 cold, circadian rhythm, and rna binding 2
AT4G39260	ATGRP8	ATGRP8 cold, circadian rhythm, and RNA binding 1
AT5G17920	ATCIMS1	ATMS1__Cobalamin-independent synthase family protein
AT3G03780	ATMS2	ATMS2 methionine synthase 2
AT5G08690		ATP synthase alpha/beta family protein
AT5G08670		ATP synthase alpha/beta family protein
AT3G26420	ATRZ-1ARBGB2	ATRZ-1A RNA-binding (RRM/RBD/RNP motifs)
AT2G30110	ATUBA1-MOS5	ATUBA1 ubiquitin-activating enzyme 1
AT5G06460	ATUBA2	ATUBA2 ubiquitin activating enzyme 2
AT3G48340	CEP2	CEP2__Cysteine proteinases superfamily protein
AT4G24800	ECIP1-MRF3	ECIP1 MA3 domain-containing protein
AT4G34200	EDA9P-GDH1	EDA9 D-3-phosphoglycerate dehydrogenase
AT1G29880		Glycyl-tRNA synthetase / glycine--tRNA ligase
AT1G18500	IPMS1-MAML-4	IPMS1 methylthioalkylmalate synthase-like 4
AT1G53240	mMDH1	mMDH1 Lactate/malate dehydrogenase family protein
AT3G04600		Nucleotidyl transferase superfamily protein
AT2G22780	PMDH1	PMDH1__peroxisomal NAD-malate dehydrogenase 1
AT2G29400	ATTOPP1	TOPP1__type one protein phosphatase 1
AT5G39320	UDG4	UDG4 UDP-glucose 6-dehydrogenase family protein
AT3G29360		
AT4G11150	UGD2	UGD2__UDP-glucose 6-dehydrogenase family protein
	VHA-E1	VHA-E1 vacuolar ATP synthase subunit E1

545

546

Table 2. Cyanilated proteins identified in cas-c1 root hair samples.

AGI	Gene alias	Annotation
AT1G18080	ATARCA	ATARCA Transducin/WD40 repeat-like superfamily protein
AT2G21660	ATGRP7	ATGRP7__cold, circadian rhythm, and rna binding 2
AT4G39260	ATGRP8	ATGRP8__cold, circadian rhythm, and RNA binding 1
AT1G27130	ATGSTU1	ATGSTU13__glutathione S-transferase tau 13
AT5G17920	ATMS1	ATMS1__Cobalamin-independent synthase family protein
AT3G03780	ATMS2	ATMS2__methionine synthase 2
AT2G20420		ATP citrate lyase (ACL) family protein
AT3G28715		ATPase, V0/A0 complex, subunit C/D
AT3G28710		ATPase, V0/A0 complex, subunit C/D
AT4G13940	ATSAHH1-HOG1	ATSAHH1__S-adenosyl-L-homocysteine hydrolase
AT3G23810	ATSAHH2	ATSAHH2__S-adenosyl-L-homocysteine (SAH) hydrolase 2
AT3G48340	CEP2	CEP2__Cysteine proteinases superfamily protein
AT2G42490	CuAOZ-zeta	CuAOZ-zeta__Copper amine oxidase family protein
AT3G53580		diaminopimelate epimerase family protein
AT1G13950	ATELF5A	ELF5A-1__eukaryotic elongation factor 5A-1
AT1G69410	ATELF5A-3	ELF5A-3__eukaryotic elongation factor 5A-3
AT1G31070	GLCNA.UT1	GLCNA.UT1__N-acetylglucosamine-1-phosphate uridylyltransferase 1
AT5G16760	AtITPK1	ITPK1__Inositol 1,3,4-trisphosphate 5/6-kinase family protein
AT5G50850	MAB1	MAB1__Transketolase family protein
AT2G33340	MAC3B	MOS4-associated complex 3B
AT4G24800	ECIP1-MRF3	MRF3__MA3 domain-containing protein
AT4G35460	ATNTRB	NTRB__NADPH-dependent thioredoxin reductase B
AT3G54960	ATPDI1	PDIL1-3__PDI-like 1-3
AT3G15000	MORF8-RIP1	RIP1__cobalt ion binding
AT4G13930	SHM4	SHM4__serine hydroxymethyltransferase 4
AT3G18060		transducin family protein / WD-40 repeat family protein
AT2G16950	ATTRN1	TRN1__transportin 1
AT5G42980	ATH3	TRXH3__thioredoxin 3
	UDG4	UDG4__UDP-glucose 6-dehydrogenase family protein
AT5G39320		
AT2G21270	UFD1	UFD1__ubiquitin fusion degradation 1
AT3G29360	UGD2	UGD2__UDP-glucose 6-dehydrogenase family protein
AT5G15490	UGD3	UGD3__UDP-glucose 6-dehydrogenase family protein

Figure legends

Fig. 1. Root meristem phenotype in wild type and *cas-cl* mutant. Col-0 (A) and *cas-cl* mutant (B) seedlings were grown for 7 d on MS medium supplemented with sucrose in vertical plates. Representative images are shown. White squares indicate QC cells. Propidium iodide was used to visualize cell walls.

Fig. 2. Root meristem size in MS medium. A) A representative root of 7-days-old wild type (wt) and *cas-cl* mutant stained with propidium iodide. White arrows indicate the transition between the elongation and transition zones in wild type and *cas-cl* mutant seedlings. B) Root meristem length of wild type and *cas-cl* mutants in MS medium. Media $\pm$ SD are shown in the graph. N>20.

Fig 3. Root length measurement in nutrient deficient media. Wild type and *cas-cl* mutant seedling were grown for 9 d in vertical plates of MS medium or MS medium in the absence of different nutrient compounds. Root length was measured with ImageJ software. Values are means $\pm$ SD of three independent experiments.

Fig. 4. GFP localization in roots from the wt-pCOBL9:GFP and *cas-cl*-pCOBL9:GFP transgenic lines. Plants were grown 6 days in MS medium. Representative images are shown.

Fig. 5. A, Venn diagram showing the unique and intersection of the identified protein in the proteomic analysis of Petricka et al, 2012 and this work. B, Venn diagram showing the unique and intersection of the identified proteins in wild type and *cas-cl* root hair samples.

Fig. 6. Bubble plot of the functional classification of the identified proteins according to the plant-specific database MapMan. Bubble columns represent the proteins classified within each bin of the total proteins identified in wild type (wt), *cas-cl* root hair samples, and those only present in wt or *cas-cl* root hair samples. Numbers besides the bubble represent the amount of proteins classified within the bin.

Fig. 7. LC-MS/MS mass spectra of the tryptic peptide containing the S-cyanlated Cys³⁴⁶ residue of the S-Adenosyl homocysteine hydrolase SAHH1/HOG1 protein. The table contains the predicted ion type for the modified peptide, and the ions detected in

584 the spectrum are highlighted in green color.

585

586 Fig. 8. LC-MS/MS mass spectra of the tryptic peptide containing the *S*-cyanlated Cys¹⁰
 587 residue of the Glycine Rich Protein 7, (GRP7). The table contains the predicted ion type
 588 for the modified peptide, and the ions detected in the spectrum are highlighted in green
 589 color.

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592

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