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Short paper

Involvement of salicylic acid in the response to potassium deficiency revealed by metabolomics

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Highlights

- Early metabolic consequences of potassium deficiency are explored using metabolomics in both wild type and triple mutant *hak5 akt1 cipk23* in Arabidopsis.
- In addition to the reorchestration of sugar metabolism, salicylic acid 2-*O*- β glucoside and salicin appear in most significant metabolites.
- It suggests that salicylic acid metabolism and signalling participate in the response to low K conditions.

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1 **Abstract**

2 Potassium (K) deficiency has consequences not only on cellular ion balance and transmembrane
3 potential but also on metabolism. In fact, several enzymes are K-dependent including enzymes in
4 catabolism, causing an alteration in glycolysis and respiration. In addition, K deficiency is associated
5 with the induction of specific pathways and accumulation of metabolic biomarkers, such as putrescine.
6 However, such drastic changes are usually observed when K deficiency is established. Here, we
7 carried out a kinetic analysis with metabolomics to elucidate early metabolic events when nutrient
8 conditions change from K-sufficiency to K-deficiency in *Arabidopsis* rosettes from both wild type and
9 mutants affected in both K absorption and low-K signalling (*hak5 akt1 cipk23*). Our results show that
10 mutants have a metabolomics pattern similar to K-deficient wild-type, showing a constitutive
11 metabolic response to low K. In addition, shifting to low K conditions induces (i) changes in sugar
12 metabolism and (ii) an accumulation of salicylic acid metabolites before the appearance of biomarkers
13 of K deficiency (putrescine and aconitate), and such an accumulation is more pronounced in mutants.
14 Our results suggest that early events in the response to low K conditions involve salicylic acid
15 metabolism.

16 17 **1. Introduction**

18 Potassium (K) is a major nutrient representing up to $\approx 5\%$ of plant matter. K is essential for plant
19 stomatal conductance and photosynthesis, phloem transport and cellular ion balance [1]. In many
20 cultivated areas where K availability is naturally low, K fertilisation is required, representing a total
21 market of \$12.6 billion at the global scale [2]. K fertilisation is of importance for high K-demanding
22 crops such as oil palm, since some tropical soil types are relatively K-poor and waterlogging can limit
23 further K assimilation [3].

24 In the past decade, intense efforts have been devoted to understand response mechanisms to
25 low K conditions and identify biomarkers of K deficiency. It is believed that low K sensing involves
26 CBL1/9 (CALCINEURIN B LIKE 1/9) and CIPK23 (CBL INTERACTING PROTEIN KINASE 23) via a Ca^{2+}
27 signal triggered by low K [4-6]. CIPK23 in turn phosphorylates and activates the voltage-gated
28 channel AKT1 [7, 8], and the transporter HAK5 [9] thereby enhancing K^+ absorption. Other targets of
29 CIPK23 include KUP4 (K^+ UPTAKE TRANSPORTER) and the vacuolar channel TPK (TWO PORES K^+)
30 (reviewed in [4]). Downstream events associated with low K conditions include a reorchestration of
31 metabolism, due to both indirect (regulatory mechanisms) and direct effects (use of K^+ as a cofactor)
32 of K^+ scarcity. Regulatory mechanisms seem to involve the central metabolic regulator mTOR, which
33 interacts effectively with CIPK23 [10]. Direct effects concern typically pyruvate kinase (EC 2.7.1.40,
34 glycolytic enzyme) and succinate thiokinase (EC 6.2.1.4, Krebs cycle enzyme), which are K^+ -
35 dependent such that their activity is inhibited when K^+ concentration is low [11-13]. K deficiency thus
36 activates alternative pathways such as the malate cycle (phosphoenolpyruvate carboxylase, malate
37 dehydrogenase, malic enzyme) to synthesize pyruvate, and the aconitate-citramalate shunt to

38 compensate for the inhibition succinate metabolism [3, 14-18]. Also, K deficiency is accompanied by
39 putrescine accumulation, sometimes to very high levels [19]. It has been proposed recently that
40 putrescine itself plays a regulatory role since putrescine addition down-regulates further the content in
41 pyruvate kinase [17]. Nevertheless, drastic metabolic changes like putrescine or aconitate build-up are
42 usually observed when plants cultivated under contrasting K conditions are compared, or when K
43 deficiency is well-established. In other words, it is possible that early metabolic events occurring when
44 K becomes progressively limiting have not been observed yet. Missing early events can be
45 problematic when monitoring the K status of crops since catching first symptoms of deficiency is
46 essential to adjust fertilization rapidly and thereby avoid consequences on yield.

47 Here, we carried out metabolic kinetics in *Arabidopsis*, using both wild-type (WT) and
48 mutants affected in potassium absorption and signalling, with emphasis on the triple mutant *hak5 akt1*
49 *cipk23* that has been characterized previously [20]. Plants were grown in hydroponics under K-
50 sufficient conditions and then either challenged under low K or kept under K-sufficient conditions
51 (Fig. S1). Metabolomics analyses were performed using GC-MS profiling. Both univariate and
52 multivariate statistics were conducted to highlight metabolites associated with genotype and low K
53 effects.

54

55 **2. Methods**

56 *2.1. Plant material, K content and Rb uptake*

57 Single, double and triple mutants were characterized in [20]. Sowing was done on 0.8% agar (Bacto)
58 and plants were grown on hydroponics under short days 8:16 h day:night. K⁺ concentration was varied
59 with KCl in nutrient solution composition. For the metabolomic kinetics experiment was designed to
60 follow early metabolic changes. Plants were grown at 5 mM KCl for 43 d and then kept at 5 mM or
61 transferred to 0.01 mM for several weeks in the nutrient solution consisting of Ca(NO₃)₂ (1.5 mM),
62 MgSO₄ (0.35 mM), NaH₂PO₄ (0.2 mM) and a mixture of microelements (Na-Fe-EDTA 0.1 mM,
63 MnSO₄ 1 μM, ZnSO₄ 1 μM, CuSO₄ 0.5 μM, H₃BO₄ 37.5 μM, Na₂MoO₄ 0.1 μM, Co(NO₃)₂ 0.1 μM).
64 Four samplings were done (7, 11, 18 and 29 days after transfer) (Fig. S1). To analyse early metabolic
65 events, only the first three samplings were used in statistics (Fig. 1). Ion transport activity of plants
66 was checked via Rb⁺ uptake assays carried out in a separate experiment. Rb⁺ uptake was measured on
67 plants grown at 10 mM KCl (with 1/5 Hoagland solution) for 30 d and transferred to 0.5 mM for 5 d,
68 using Rb⁺ addition to a final concentration of 0.5 mM. K and Rb content in plants was measured by
69 ICP-OES (Perkin-Elmer).

70

71 *2.2. Metabolomics*

72 Plant leaves were snap-frozen in liquid nitrogen, frozen-dried and ground in fine powder.
73 Metabolomics analyses were carried out by GC-MS on water:methanol (90:10 v:v) extracts
74 derivatized by trimethylsilylation in pyridine, using ribitol as an internal standard. Data analysis were

75 performed using Metabolome Express (www.metabolome-express.org). Further details are provided in
76 [3] and in Supplementary Notes S1. Statistical multivariate OPLS analyses were carried out with
77 Simca 16 (Umetrics) as in [17] using metabolites as X variables and genotype, time and K as Y
78 variables.

79

80 **3. Results**

81 *3.1. Plant growth*

82 Thirty-five days after germination, there were little differences in plant size between genotypes (Fig.
83 S2a) showing that high K conditions used here were K-sufficient. Four weeks after having shifted
84 conditions to low K, observed growth was very limited, with an increase of $\approx 60\%$ only in rosette
85 diameter in the WT. As expected, growth was nearly arrested in the double *hak5 akt1* and triple *hak5*
86 *akt1 cipk23* mutants (Fig. S2b). The single mutant *cipk23* did not show a significant decrease in rosette
87 size compared to the WT under low K but exhibited a smaller rosette under high K, suggesting that
88 CIPK23 is involved in the growth response to high K availability.

89

90 *3.2. Potassium content and uptake*

91 As expected, net potassium uptake capacity (estimated using Rb^+ uptake assays) of the double *hak5*
92 *akt1* and triple *hak5 akt1 cipk23* mutants was effectively 60% lower than that of the WT (Fig. S3a).
93 When challenged under low K, all plants were depleted in K in shoots and roots (Fig. S3b-c), with a
94 significantly lower content in mutants compared to WT (up to 30% lower). Interestingly, there was no
95 significant difference in uptake rate and potassium content between the two mutants, in agreement
96 with the same rosette size under low K (Fig. S2) (unlike results found in [20]).

97

98 *3.3. Metabolic signature*

99 Metabolomics analyses led to the identification and quantification of 204 analytes. Statistics carried
100 out by supervised multivariate analysis (OPLS) allowed facile discrimination of sample types, with
101 respect to genotype and K conditions (Fig. 1a). In fact, the score plots showed a discrimination of K
102 conditions along axis 1, time along axis 2 and genotype along axis 3. The statistical model was highly
103 explicative ($R^2 = 0.847$) and robust ($Q^2 = 0.516$). A univariate analysis by ANOVA was also
104 conducted and best discriminant metabolites are shown in a volcano plot (Fig. 1b) and a heatmap (Fig.
105 1c). Most genotype-significant metabolites were sugars, with hexoses and polyols increased (cluster 2,
106 Fig. 1c) and oxidised derivatives of sugars (galacturonate, erythronate and 2-oxoglucose) decreased
107 (cluster 1, Fig. 1c) in the *hak5 akt1 cipk23* mutant. Interestingly, only three non-sugar metabolites
108 were significant for the genotype effect: serine, cysteine and salicylate glucoside (salicylic acid 2-O- β
109 glucoside). Metabolites associated with a genotype \times K interaction effect (Fig. 1d) were mostly sugars,
110 with three exceptions: succinate (increased in the triple mutant early on), serine and salicin (increased
111 in the triple mutant later on). Interestingly, most discriminant metabolites associated with the K effect

112 were similar to those significant for the genotype effect (Fig. S4a-b): typically sugars and polyols (as
113 well as salicylate glucoside and salicin) increased while oxidised derivatives (galacturonate,
114 erythronate) decreased under low K. In other words, the metabolic effect of the triple mutation was
115 rather similar to that of low K availability, confirming that mutants experienced constitutive low K
116 cellular conditions.

117 Salicylate glucoside and salicin accumulated under low K, to a higher extent in the triple
118 mutant (Fig. S4c). Also, salicylic glucoside was significantly higher at the first sampling time (7 d
119 after having switched K conditions), even before biomarkers of K deficiency, aconitate and putrescine,
120 accumulated (Fig. S4a). This resulted in putrescine being much less significant for the K effect
121 compared to salicylate glucoside (in Fig. S4b, note that putrescine was just above the Bonferroni
122 threshold). It is also worth noting that while salicylate glucoside increased with time under low K,
123 other phenylpropanoids significant for the K effect (3,4,5-trihydroxybenzoate, sinapate) declined with
124 time, suggesting rerouting of the entire phenylpropanoid pathway in favour of salicylic acid (SA)
125 production.

126

127 **4. Discussion**

128 *4.1. Alteration of sugar metabolism under low K*

129 Early metabolic changes caused by low K and genotype mostly concerned sugars, with an increase in
130 hexoses, polyols and pentoses and a decrease in metabolites coming from sugar oxidation (summary
131 provided in Fig. S5). Also, low K conditions led to a decrease in organic acids such as citrate and
132 fumarate, while succinate increased in the triple mutant under low K (Fig. 1c). This rapid and
133 concerted effect on sugar and organic acids when conditions are switched from K sufficiency to K
134 deficiency probably reflects the direct effect of low K^+ cellular concentration, inhibiting (i) phloem
135 export from leaves thereby causing a general increase in sugars and myoinositol [18]; (ii) enzyme
136 activities of catabolism that are K-dependent (see *Introduction*). It is worth noting that while citrate
137 and fumarate were amongst most significantly decreased metabolites, aconitate was more abundant
138 under low K, suggesting the involvement of the aconitate-citramalate shunt as observed previously in
139 sunflower and oil palm [3, 16]. Unlike sugars, putrescine did not increase much at the beginning of the
140 low K response and it took two weeks to observe putrescine build-up. This effect might have come
141 from the lag time required to induce arginine decarboxylase (the source of putrescine in *Arabidopsis*).

142

143 *4.2. Salicylic acid metabolism is part of the low K response*

144 A striking feature of the metabolic signature observed both under low K and in the triple mutant *hak5*
145 *akt1 cipk23* is the appearance of SA derivatives: salicylate glucoside and salicin. Interestingly,
146 salicylate glucoside increased considerably early on in the triple mutant (while salicin increased
147 significantly later). It is possible that salicylate glucoside appeared at the expense of other forms of
148 conjugated forms of salicylic acid not detected here by GC-MS. However, since other

149 phenylpropanoids declined it is plausible that there was a reorientation of phenylpropanoid
150 metabolism towards SA biosynthesis. There are two major routes for SA synthesis, involving either
151 isochlorogenic acid or cinnamic acid [21]. Here, it is likely that it involved the isochlorogenic pathway
152 since sinapic acid was affected by K conditions and furthermore, in sunflower proteome under K
153 deficiency, a significant decrease in cinnamyl alcohol dehydrogenase and coumarate CoA ligase was
154 found [17]. Salicylate glucoside and salicin are synthesized by UDP-glucosyl transferases, transported
155 by several sucrose transporters, stored in vacuoles and represent a pool of remobilizable SA [21-24].

156 The increase in the SA pool found here maybe reflects the involvement of SA signalling under
157 low K stress. SA is generally related to pathogenesis-related signalling but has been found to
158 participate in the response to salt stress, drought and heavy metal (in particular cadmium) [21, 25, 26].
159 Interestingly, in sunflower proteome under K deficient, there is an increase in benzyl alcohol *O*-
160 benzoyl transferase [17], which is involved in SA metabolism and has been found to be up-regulated
161 during the hypersensitive reaction to pathogens [27]. Also, the addition of SA has been found to
162 alleviate symptoms of K deficiency on plant morphology [28]. A NPR1-independent pathway of SA
163 signalling has been shown to be essential during salt and oxidative stress [29]. Here, a similar pathway
164 is perhaps used under K deficiency.

165

166 **5. Conclusions and future directions**

167 Our results show that aside strong effects on sugar metabolism and catabolism, early responses to K
168 deficiency have consequences on SA metabolism, and this is particularly visible in the triple mutant
169 *hak5 akt1 cipk23*. Future studies are warranted to provide more insight on molecular mechanisms
170 linking low K to the activation of SA metabolism and reveal specific roles of SA in the response to
171 low K conditions.

172

173 **Contributions**

174 G.T. and J.C. designed the experiment; F.R. and M.N.C. provided seeds and performed mineral
175 analyses; J.C. set up the experiments and did metabolomics analyses; G.T. did data integration and
176 wrote the first draft of the paper; all authors contributed to the final version of the paper.

177

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187

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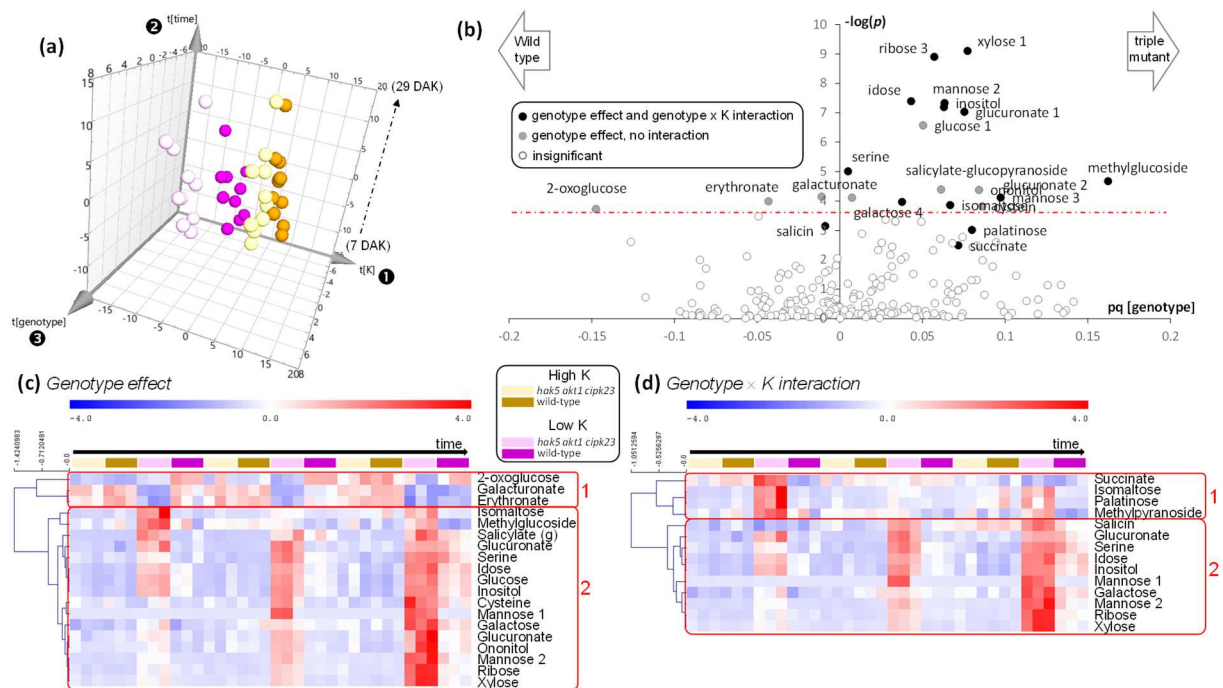


Fig. 1. Metabolomics pattern of wild-type and triple mutant *hak5 akt1 cipk23* Arabidopsis rosettes: genotype effect when plants are challenged with low (0.01 mM) or high (5 mM) KCl in hydroponics after 43 days under high K. (a) Score plot of the multivariate analysis (OPLS) showing facile discrimination of samples with K conditions (axis 1), time (axis 2) and genotype (axis 3). Along axis 2, time goes from bottom (7 days after having switched K conditions) to top (29 days). (b) Volcano plot representing best biomarker metabolites associated with the genotype effect, using the loading in the OPLS analysis (x axis) against the logarithm of the p-value in univariate analysis (ANOVA; y axis). The horizontal dash-dotted line stands for the Bonferroni threshold (here, $-\log(p) = 3.61$). (c) Heatmap showing significant metabolites in ANOVA for the genotype effect. Hierarchical clustering (Euclidean distance) is shown on left and clusters are labelled with red frames and numbers. (d) Heatmap showing significant metabolites in ANOVA for the genotype \times K interaction, with also a hierarchical clustering on left. In (c), "salicylate (g)" stands for salicylate glucoside as it appears in (b). In both (c) and (d), numbers along with metabolites refer to distinct derivatives in GC-MS analyses. The description of the specific effect of K is provided in Fig. S4.