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Version of Record: https://www.sciencedirect.com/science/article/pii/S0981942821001868
Manuscript_2b620e7954dff3aec815e8b022399c85

*Short paper*

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

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**Keywords:** Potassium; deficiency; metabolomics; salicylic acid; CBL-CIPK regulation

## 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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**Abstract**

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

## 1. Introduction

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

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

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

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

## 2. Methods

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

Single, double and triple mutants were characterized in [20]. Sowing was done on 0.8% agar (Bacto) and plants were grown on hydroponics under short days 8:16 h day:night. $K^+$ concentration was varied with KCl in nutrient solution composition. For the metabolomic kinetics experiment was designed to follow early metabolic changes. Plants were grown at 5 mM KCl for 43 d and then kept at 5 mM or transferred to 0.01 mM for several weeks in the nutrient solution consisting of $Ca(NO_3)_2$ (1.5 mM), $MgSO_4$ (0.35 mM), $NaH_2PO_4$ (0.2 mM) and a mixture of microelements (Na-Fe-EDTA 0.1 mM, $MnSO_4$ 1 µM, $ZnSO_4$ 1 µM, $CuSO_4$ 0.5 µM, $H_3BO_4$ 37.5 µM, $Na_2MoO_4$ 0.1 µM, $Co(NO_3)_2$ 0.1 µM). Four samplings were done (7, 11, 18 and 29 days after transfer) (Fig. S1). To analyse early metabolic events, only the first three samplings were used in statistics (Fig. 1). Ion transport activity of plants was checked via $Rb^+$ uptake assays carried out in a separate experiment. $Rb^+$ uptake was measured on plants grown at 10 mM KCl (with 1/5 Hoagland solution) for 30 d and transferred to 0.5 mM for 5 d, using $Rb^+$ addition to a final concentration of 0.5 mM. K and Rb content in plants was measured by ICP-OES (Perkin-Elmer).

### *2.2. Metabolomics*

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

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

## 3. Results

### *3.1. Plant growth*

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

### *3.2. Potassium content and uptake*

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

### *3.3. Metabolic signature*

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

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

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

## 4. Discussion

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

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

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

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

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

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

## 5. Conclusions and future directions

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

## Contributions

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

## Acknowledgements

G.T. thank the *Région Pays de la Loire* and *Angers Loire Métropole* for its financial support via the grant Connect Talent Isoseed. We thank Sergio García for his skilful assistance. This work was funded by grant number 20806/PI/18 (to FR) from Fundación Séneca de la Región de Murcia, Spain, grant PID2019-106649RB-I00 from Ministerio de Ciencia e Innovación, Spain (to FR and MN-C). This work was also supported by the Red de Excelencia BIO2016-81957-REDT from Ministerio de Economía, Industria y Competitividad. All grants from Spanish ministries were co-financed by the

FEDER Fund. MN-C, is recipient of a Ramón y Cajal Fellowship (RyC-2017-21924) from Ministerio de Economía y Competitividad, Spain.

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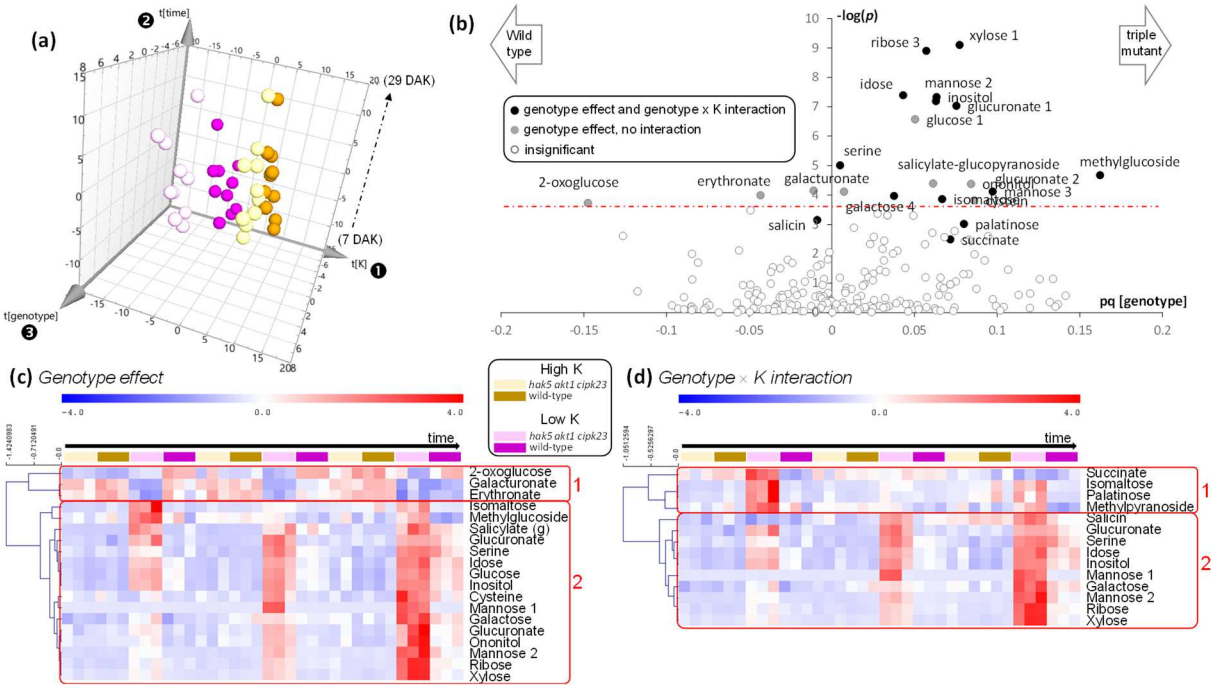


**Fig. 1. Metabolomics pattern of wild-type and triple mutant *hak5 akt1 cipk23* Arabidopsis rosettes: genotype effect** when plants are challenged with low (0.01 mM) or high (5 mM) KCl in hydroponics after 43 days under high K. (a) Score plot of the multivariate analysis (OPLS) showing facile discrimination 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 × 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.