Proteome quantification of cotton xylem sap suggests the mechanisms of potassium-deficiency-induced changes in plant resistance to environmental stresses

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Proteomics was employed to investigate the molecular mechanisms of apoplastic response to potassium (K)-deficiency in cotton. Low K (LK) treatment significantly decreased the K and protein contents of xylem sap. Totally, 258 peptides were qualitatively identified in the xylem sap of cotton seedlings, of which, 90.31% were secreted proteins. Compared to the normal K (NK), LK significantly decreased the expression of most environmental-stress-related proteins and resulted in a lack of protein isoforms in the characterized proteins. For example, the contents of 21 Class III peroxidase isoforms under the LK were 6 to 44% of those under the NK and 11 its isoforms were lacking under the LK treatment; the contents of 3 chitinase isoforms under LK were 11–27% of those under the NK and 2 its isoforms were absent under LK. In addition, stress signaling and recognizing proteins were significantly down-regulated or disappeared under the LK. In contrast, the LK resulted in at least 2-fold increases of only one peroxidase, one protease inhibitor, one non-specific lipid-transfer protein and histone H4 and in the appearance of H2A. Therefore, K deficiency decreased plant tolerance to environmental stresses, probably due to the significant and pronounced decrease or disappearance of a myriad of stress-related proteins.

Potassium is a macronutrient that participates in many physiological processes, such as osmotic adjustment, photosynthesis, transport and enzyme activation in plants. Potassium deficiency can directly lower various crop plant productivities and qualities, which may be indirectly reduced via a combination of biotic and abiotic stresses.

In general, a high K status in crops decreases the incidence of diseases and pests. For example, in K-deficient soils, cotton and other crops can be susceptible to Fusarium wilt and root rot caused by Fusarium oxysporum sp. The application of K either before or after planting is equally effective in reducing this incidence. In rice, increased K supply results in increased resistance to brown leaf spot disease and bacterial leaf blight. Similarly, higher K supply successfully suppresses disease incidence in soybean and wheat. Improving the K nutritional status of plants may be very important for the survival of crop plants under abiotic stress conditions, such as drought, chilling, salt stress and high light intensity. For example, frost damage is inversely related to the available K content in soils and the K concentration in potato leaves; potassium fertilization increases frost resistance in the three K-availability soils, particularly for the soil with the lowest K status. Similar effects were reported by Sharma and Sud. Hakerlerler et al. observed that increasing the amount of

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Potassium-deficient treatment affected mineral nutrient contents. Emerging cotton seedlings in wet sand were transferred to a normal solution, grown for 3 d, and separated into a K-deficient solution and a new normal solution and grown for 7 d. These cotton seedlings were used for organ and xylem sap sampling and index determination. For each organ, means within each column followed by the * and ** are significantly different according to t-test, respectively, p < 5% and p < 1%, n = 6.

| Organs          | Treatment | K  | Ca   | Mg   | Fe   | Cu   | Zn   |
|-----------------|-----------|----|------|------|------|------|------|
|                  |           | mg g⁻¹ dry weight |     |      |      |      |      |
| Root            | NK        | 35.25** | 30.85 | 13.42 | 1.74 | 0.26 | 0.82 |
|                 | LK        | 20.27  | 58.75  | 53.39  | 3.78 | 0.57* | 2.74* |
| Cotyledon       | NK        | 20.37** | 72.24 | 23.93 | 0.73 | 0.14 | 0.34 |
|                 | LK        | 12.95  | 100.81** | 44.44 | 1.88 | 0.30* | 0.85** |
| Forth true leaf | NK        | 36.69** | 70.96 | 28.13 | 1.89 | 0.40 | 0.95 |
|                 | LK        | 22.17  | 104.76* | 41.61** | 2.45* | 0.43 | 0.99 |
| Xylem sap       | NK        | 511.31** | 305.83 | 50.32 | 1.61 | 0.08 | 1.70 |
|                 | LK        | 66.41  | 429.95** | 79.35** | 1.20 | 0.11 | 2.65** |

Table 1. Effects of potassium deficiency on the mineral nutrient contents. Emerging cotton seedlings in wet sand were transferred to a normal solution, grown for 3 d, and separated into a K-deficient solution and a new normal solution and grown for 7 d. These cotton seedlings were used for organ and xylem sap sampling and index determination. For each organ, means within each column followed by the * and ** are significantly different according to t-test, respectively, p < 5% and p < 1%, n = 6.

K fertilizer increases low-temperature stress tolerance, resulting in as much as 2-fold increases in the yield for various non-greenhouse-grown vegetable crops (tomato, pepper, and eggplant) at temperatures of from 4–16 °C.

The apoplast, which includes the xylem sap, has received specific attention because it constitutes the first barrier to biotic and abiotic stresses. This occurs through defense, recognition, and signaling information to cells for further response. Several research groups have characterized the processes related to plant defense to biotic stresses and abiotic stresses by analyzing the apoplastic protein fractions.

In general, potassium deficiency can decrease both biotic and abiotic resistance abilities, though there is currently no analysis of the proteome in the apoplast to corroborate these decreases. Understanding how these decreases occurs and the corresponding mechanisms involved are important for preventing problems associated with lowered resistance. This understanding also provides a good reference point for increasing plant resistance to biotic and abiotic stresses under potassium-deficient conditions. Previous work has demonstrated that many proteins related to environmental stress are found in cotton xylem sap. The aim of this study is to qualitatively and quantitatively analyze changes in xylem sap proteins, especially proteins that are related to biotic and abiotic stresses under potassium-deficient conditions, and to further investigate the mechanisms controlling the decreased potassium-deficiency-induced defense ability.

Results

Potassium-deficient treatment affected mineral contents, physiological traits and cotton seedling growth. When cotton seedlings were first cultivated under normal K levels for 3 d, subsequent potassium deficiency for 7 d significantly decreased the K content in the root, old leaf (cotyledon) and new leaf (forth true leaf) components, although substantial increases in the micronutrient (Ca, Mg, Fe, Cu, and Zn) contents in corresponding organs were found; however, there was a non-significant change in Cu and Zn in the new leaf component. It addition, this cultivation significantly decreased the K concentration and significantly increased the Ca, Mg and Zn concentrations in the xylem sap (Table 1).

The potassium deficiency of the plant significantly decreased the soluble protein content, the activities of guaiacol-peroxidase (GPX; Class III peroxidase; EC1.11.1.7) and superoxide dismutase (SOD; EC1.15.1.1), and pH in the xylem sap, although a significant increase in the xylem sap volume, probably resulting from high root pressure induced by the decreased K content, was also found (Table 3 and supplementary Fig. S2). Under prolonged K deficiency, the growth of cotton seedlings was gradually inhibited. At 5 d of K deficiency, the root length and surface area began to significantly decrease compared to potassium-sufficient plants. At 7 d, the potassium-deficient plants had significantly reduced leaf area, plant height, dry, leaf, stem, root weights, compared to the potassium-sufficient plants (Table 2; Fig. 1).

General situation of qualitative and quantitative proteins in the cotton xylem sap. In total, 258 qualitative peptides were identified in the xylem sap of the cotton seedlings, including 72 uncharacterized...
proteins. Among the qualitative peptides, 116 catered to the conditions of quantitative peptides based on a comparison between the low K (LK) and normal K (NK) treatments; 41 and 5 proteins were not detectable in the LK and NK treatments, respectively (Table 4).

Proteins that had ≥2 detection signals in three NK replicates and no detection signal in three LK replicates were defined as being non-detectable in LK (NLK) (A). Proteins with ≥2 detection signals in three LK replicates and no detection signal in three NK replicates were defined as being non-detectable in NK (NNK) (B). There were 56 proteins with no detection signal in three LK replicates and no detection signal in three NK replicates.

Figure 1. Photograph of comparative growth performances and morphological data of cotton seedlings after different days of LK treatments along with control. Emerging cotton seedlings in wet sand were transferred into a normal solution, grown for 3 d, and separated into a K-deficient solution and a new normal solution and grown for 7 d. Left (A): photograph of comparative growth performances of cotton seedlings after 1, 3, 5, 7 d of LK treatments along with control; Right: morphological data of cotton seedlings after 1, 3, 5, 7 d of LK treatments along with control (B): Leaf area; (C): Plant height; (D): Root length; (E): Root surface area.
These proteins may have been caused by no concentration or a low concentration of quantitative peptides. Moreover, there were 28 proteins with only one detection signal in three NK replicates and no detection signal in three LK replicates. There were 4 proteins with only one detection signal in three NK replicates and ≥1 in comparison.

| Identified proteins | Qualitative peptides | Quantitative peptides in comparison | Non-quantitative peptides in comparison |
|--------------------|----------------------|-------------------------------------|----------------------------------------|
| Total              | 258                  | 116                                 | 41 5 96                                 |
| Uncharacterized    | 72                   | 21                                  | 9 4 38                                  |

Table 4. Qualification and quantification of xylem sap proteins. Emerging cotton seedlings in wet sand were transferred into a normal solution, grown for 3 d, and separated into a K-deficient solution and a new normal solution and grown for 7 d. These cotton seedlings were used for xylem sap sampling, and the identified proteins not meeting the comparative quantification requirements were statistically classified.

| Secretd proteins | Qualitative peptides | Quantitative peptides in comparison | Non-quantitative peptides in comparison |
|------------------|----------------------|-------------------------------------|----------------------------------------|
| Total            | 233                  | 109                                 | 38 4 82                                 |
| Classical        | 147                  | 80                                  | 31 1 35                                 |
| Non-classical    | 86                   | 29                                  | 7 3 47                                 |

Table 5. Classical and non-classical secreted proteins based on the proteins identified in the cotton seedling xylem sap. The identified proteins were predicted as classical secreted proteins using SignalP or non-classical secreted proteins using SecretomeP. More details can be found in Supplementary Tables 1 and 2. Note: NLK: being non-detectable in low K; NNK: being non-detectable in normal K.

Figure 2. Xylem sap proteins that were qualified but did not meet the comparative quantification requirements between the normal K and low K treatments. Emerging cotton seedlings in wet sand were transferred into a normal solution, grown for 3 d, and separated into a K-deficient solution and a new normal solution and grown for 7 d. These cotton seedlings were used for xylem sap sampling, and the identified proteins not meeting the comparative quantification requirements were statistically classified.

(C). These proteins may have been caused by no concentration or a low concentration of quantitative peptides. Moreover, there were 28 proteins with only one detection signal in three NK replicates and no detection signal in three LK replicates (D). There were 4 proteins with only one detection signal in three NK replicates and ≥1 detection signal in comparison.
Identification of classical and non-classical secreted proteins in cotton xylem sap. The proportion of secreted proteins within the total was 90.31%; for classical peptides (predicted with a signal peptide by SignalP), the ratio was 56.98%. For the quantitative peptides in comparison, the rate was 93.97%, and the ratio for classical peptides was 68.97% (Table 5). The proteins that were not predicted as being secreted in the latter category (predicted as another type according to TargetP) included 5 uncharacterized proteins and secreted protein isoforms, such as "O-glycosyl hydrolyases family 17 protein isoform 1" and "glycosyl hydrolyase superfamily protein isoform 3"; two protein fragments were found, i.e., "peroxidase 6 (Fragment)" and "chaperonin CNP60-like protein (Fragment)"; lactoylglutathione lyase; and histone 4 (Table 6, Supplemental Table 1) were also found.

Protein classification and their regulation by potassium deficiency in the xylem sap. For the quantitative or lacking proteins in the LK or NK treatments, identified proteins were classified as pathogenesis-related (PR) proteins, oxido-reduction-related proteins, signaling proteins, other stress-related proteins, cell wall metabolism proteins, proteins with interacting domains, miscellaneous proteins and uncharacterized proteins. PR proteins were dominant, including antifungal protein (PR-1), β-1, 3-glucanases (PR-2), chitinases (PR-3 -4, 8, and 11), thaumatin-like proteins (PR-5), protease inhibitors (PR-6), endo-proteases (PR-7), peroxidases (PR-9), and non-specific lipid transfer proteins (PR-14). Other stress-related proteins included heparanase (putative), chaperonin CNP60-like protein (fragment), lactoylglutathione lyase, and histones. Heparanase was previously found in apoplastic fluid (AF) of poplar, maize and grapevine16 and is indirectly involved in 

Evolutionary relationship of peroxidases (EC1.11.7) in the xylem sap. Thirty-nine peroxidases in the xylem sap were identified and analyzed according to their evolutionary relationship. Phylogenetic analysis showed that the significantly decreased and non-detectable peroxidases were phylogenetically closer to each other, although the proteins that were not significantly changed were located in the same clades with some of the peroxidases (Fig. 3). Coordination of protein (listed in Table 6) expression and corresponding gene expression. Com...
| Classification | Accession No. | Protein name | Plant species | Theoretical MW/pI | Secrete traits | FC |
|----------------|--------------|--------------|---------------|-------------------|---------------|----|
| PR-1 | A0A061DWT3 | Basic pathogenesis-related protein 1 | Theobroma cacao | 24.2/4.85 | NCSP | 0.21 |
| 1,3-beta-glucosidase | E7CQ9 | GLU | Gossypium hirsutum | 50.3/5.15 | CSP | 0.44 |
| | B9RK7F | Glucan endo-1,3-beta glucosidase, putative | Ricinus communis | 55.7/6.40 | CSP | 0.18 |
| | A0A061GIR3 | O-Glycosyl hydrolases family 17 protein isoform 1 | Theobroma cacao | 53.1/4.98 | NCSP | 0.32 |
| | P93153 | 1,3-beta-glucanase | Gossypium hirsutum | 37.6/5.03 | CSP | NLK |
| | A0A061GVZ6 | O-Glycosyl hydrolases family 17 protein isoform 1 | Theobroma cacao | 53.7/8.01 | NLK |
| Chitinase | D7RT7U | Class I chitinase | Gossypium hirsutum | 34.7/6.66 | CSP | 0.21 |
| | P93154 | Chitinase | Gossypium hirsutum | 28.8/6.25 | CSP | 0.27 |
| | E5FQ62 | Class 3 chitinase | Hipsphae rhamnoide | 31.8/9.05 | CSP | NLK |
| | L7NHI5 | Class IV chitinase | Gossypium barbadense | 28.6/4.85 | CSP | NLK |
| PR-5 | Q2HFJ3 | Osmotin-like protein | Gossypium hirsutum | 26.5/7.68 | CSP | 0.07 |
| Protease inhibitor | I7GID4 | Proteinase inhibitor | Gossypium arboreum | 7.5/4.95 | NCSP | 7.15 |
| Proteases | Q6WMU5 | Polygalacturonase-inhibiting protein | Gossypium barbadense | 37.1/8.32 | CSP | 0.38 |
| | V4TEG7 | Carboxypeptidase (serine-type) | Citrus clementina | 54.0/4.99 | CSP | 0.11 |
| | G7IU18 | Subtilisin-like serine protease | Medicago truncatula | 81.7/8.88 | CSP | 0.35 |
| | B9RN88 | Aspatic proteinase nepenthesin-2, putative | Ricinus communis | 48.8/19.19 | CSP | 0.15 |
| | A0A061E9G1 | Xylem cysteine peptidase 1 | Theobroma cacao | 39.1/5.66 | NCSP | 0.20 |
| | A0A061GL56 | Cysteine proteinsases superfamily protein | Theobroma cacao | 28.4/8.50 | CSP | 0.30 |
| Peroxidases | A0A061ET17 | Peroxidase superfamily protein | Theobroma cacao | 37.4/6.50 | CSP | 0.06 |
| | V9PHD4 | Peroxidase | Populus tomentosa | 37.8/6.66 | CSP | 0.06 |
| | Q570F | Peroxidase ATP4a (Fragment) | Arabidopsis thaliana | 24.6/4.45 | NCSP | 0.10 |
| | Q8BRP3 | Apoplastic anionic gaicol peroxidase | Gossypium hirsutum | 37.4/4.60 | CSP | 0.11 |
| | A0A067TFQ4 | Peroxidase | Jatropha curcas | 36.2/5.22 | CSP | 0.11 |
| | Q6UNK7 | Peroxidase | Gossypium hirsutum | 34.9/7.75 | CSP | 0.11 |
| | A0A061DQ02 | Peroxidase superfamily protein | Theobroma cacao | 35.6/4.95 | CSP | 0.12 |
| | G7KF2 | Peroxidase | Medicago truncatula | 35.8/4.91 | CSP | 0.13 |
| | A0A058QZQ8 | Peroxidase | Eucalypytus grandis | 35.5/3.21 | CSP | 0.15 |
| | Q4W18 | Basic peroxidase | Zinnia violacea | 34.2/3.82 | CSP | 0.16 |
| | Q7XY7 | Peroxidase | Gossypium hirsutum | 35.4/4.90 | CSP | 0.3 |
| | G6TF32 | Peroxidase | Gylene max | 34.5/9.14 | CSP | 0.33 |
| | C9WF04 | Peroxidase | Gossypium hirsutum | 35.3/7.68 | CSP | 0.35 |
| | C9WF05 | Peroxidase | Gossypium hirsutum | 34.0/8.33 | CSP | 0.40 |
| | A0A061E9R2 | Caticomic peroxidase 2 | Theobroma cacao | 39.7/17.47 | NCSP | 0.44 |
| | M5WMM3 | Peroxidase | Prunus persica | 35.7/7.29 | CSP | 2.65 |
| | A0A059AI1F | Peroxidase | Eucalypytus grandis | 33.9/8.92 | CSP | NLK |
| | A0A061GTV9 | Peroxidase superfamily protein | Theobroma cacao | 36.8/9.54 | CSP | NLK |
| | B9MX6 | Peroxidase | Populus trichocarpa | 36.4/9.69 | CSP | NLK |
| | B3RB5 | Putative secretory peroxidase | Catharanthus roseus | 35.3/9.10 | CSP | NLK |
| | G7MV9 | Peroxidase family protein | Medicago truncatula | 37.6/9.34 | CSP | NLK |
| | G7MV9 | Caticomic peroxidase | Medicago truncatula | 34.7/7.70 | CSP | NLK |
| | B9SXK5 | Peroxidase 53, putative | Ricinus communis | 35.1/46.3 | CSP | NLK |
| | A0A061ENE1 | Peroxidase 24, putative | Theobroma cacao | 36.4/9.71 | CSP | NLK |
| | M1JU2 | Peroxidase 2 | Pyrus communis | 34.5/9.99 | CSP | NLK |
| | D4NYQ9 | Peroxidase | Brugueira | 36.4/10.37 | CSP | NLK |
| | X5GE19 | Peroxidase | Carica papaya | 37.9/6.73 | CSP | NLK |
| Lipid-transfer protein | Q9M686 | Non-specific lipid-transfer protein | Gossypium hirsutum | 11.9/8.72 | CSP | 2.43 |
| Oxido-reduction-related proteins | F4AYW2 | Copper binding protein | Gossypium hirsutum | 17.8/4.30 | CSP | 0.33 |
| | A0A061ECX6 | Cupredoxin superfamily protein | Theobroma cacao | 22.2/7.97 | NCSP | 0.48 |
| | A0A067FQG2 | Superoxide dismutase [Cu-Zn] | Citrus sinensis | 28.9/7.22 | CSP | 2.00 |
| | Q6TDS6 | Laccase | Gossypium hirsutum | 63.3/6.73 | CSP | NLK |
| | A0A0763ZJ6 | BnaC07g17890D protein (UDP-N-acetylmuramate dehydratase activity) | Brassica napus | 61.0/7.99 | CSP | NLK |
| Signaling proteins | A9XTL5 | Fasciclin-like arabinogalatan protein 10 | Gossypium hirsutum | 44.3/6.73 | CSP | NLK |
| | A9XTL7 | Fasciclin-like arabinogalatan protein 12 | Gossypium hirsutum | 42.9/6.34 | CSP | 0.15 |

Continued
### Table 6. Effects of K deficiency on the contents of the classified proteins in the cotton xylem sap with ≥2- or ≤0.5-fold change in comparison between LK and NK and on proteins lacking in either the LK or NK treatment.

| Classification | Accession No. | Protein name | Plant species | Theoretical MW/pI | Secretr traits | FC |
|----------------|---------------|--------------|---------------|-------------------|----------------|----|
| PR-1           | A0A061GBZ8    | Receptor-like protein kinase-related family protein | Theobroma cacao | 26.7/6.47         | CSP 0.49*      |    |
|                | A0A061EHJ2    | NSF-interacting kinase 1 | Theobroma cacao | 71.6/8.04        | CSP NLK       |    |
|                | A0A061GG78    | Cysteine-rich RLK 29 (protein serine/threonine kinase activity) | Theobroma cacao | 149.6/5.32       | CSP NLK       |    |
|                | G70F7         | Lorelei-like-GPI-anchored protein | Medicagio truncatula G | 18.6/5.61      | CSP NLK       |    |
|                | B2ZAQ1        | Peptidoglycan-binding LysM domain-containing related protein | Gossypieris kirkii | 47.3/5.30      | CSP NLK       |    |
|                | B9RP09        | Heparanase, putative | Ricinus communis | 57.3/7.49       | NCSP NLK      |    |
|                | I3T0C3        | Histone H4 | Medicagio truncatula | 11.4/12.01 | 7.54**        |    |
|                | W9RXK9        | Histone H2A | Morus notabilis | 47.3/10.69 | NCSP NNK      |    |

### Table 7. Number of different expression patterns for the different classified proteins in the cotton xylem sap under the LK and NK treatments based on the results presented in Supplemental Table 1.

| Classification          | SDR | NLK | SUR | NNK | NSC | SUM |
|-------------------------|-----|-----|-----|-----|-----|-----|
| PR-1                    | 1   | 0   | 0   | 0   | 0   | 1   |
| 1,3-beta-glucosidase    | 3   | 2   | 0   | 0   | 1   | 6   |
| Chitinases              | 3   | 2   | 0   | 0   | 1   | 6   |
| PR-5                    | 1   | 0   | 1   | 2   | 4   |
| Protease inhibitor      | 2   | 0   | 1   | 0   | 3   |
| Proteases               | 5   | 0   | 0   | 0   | 1   | 6   |
| Peroxidases             | 15  | 11  | 2   | 0   | 11  | 39  |
| Lipid transfer protein  | 0   | 0   | 4   | 0   | 1   | 5   |
| Oxido-reductases        | 3   | 2   | 1   | 0   | 5   | 11  |
| Other stress related protein | 0   | 1   | 2   | 1   | 2   | 6   |
| Signaling               | 3   | 5   | 0   | 0   | 5   | 13  |
| Cell wall metabolism    | 4   | 8   | 1   | 0   | 5   | 18  |
| Proteins with interacting domains | 2   | 0   | 0   | 0   | 1   | 3   |
| Miscellaneous           | 3   | 0   | 0   | 0   | 3   | 6   |
| Uncharacterized proteins| 10  | 10  | 3   | 4   | 8   | 35  |

### Table 8. Proteins with interacting domains.

| Classification          | Accession No. | Protein name | Plant species | Theoretical MW/pI | Secretr traits | FC |
|-------------------------|---------------|--------------|---------------|-------------------|----------------|----|
| PR-1                    | A0A061GBZ8    | Receptor-like protein kinase-related family protein | Theobroma cacao | 26.7/6.47         | CSP 0.49*      |    |
|                | A0A061EHJ2    | NSF-interacting kinase 1 | Theobroma cacao | 71.6/8.04        | CSP NLK       |    |
|                | A0A061GG78    | Cysteine-rich RLK 29 (protein serine/threonine kinase activity) | Theobroma cacao | 149.6/5.32       | CSP NLK       |    |
|                | G70F7         | Lorelei-like-GPI-anchored protein | Medicagio truncatula G | 18.6/5.61      | CSP NLK       |    |
|                | B2ZAQ1        | Peptidoglycan-binding LysM domain-containing related protein | Gossypieris kirkii | 47.3/5.30      | CSP NLK       |    |
|                | B9RP09        | Heparanase, putative | Ricinus communis | 57.3/7.49       | NCSP NLK      |    |
|                | I3T0C3        | Histone H4 | Medicagio truncatula | 11.4/12.01 | 7.54**        |    |
|                | W9RXK9        | Histone H2A | Morus notabilis | 47.3/10.69 | NCSP NNK      |    |

Table 6. Effects of K deficiency on the contents of the classified proteins in the cotton xylem sap with ≥2- or ≤0.5-fold change in comparison between LK and NK and on proteins lacking in either the LK or NK treatment. Note: LK: low K; NK: normal K; NLK: being non-detectable in LK; NNK: being non-detectable in NK; FC: fold change; CSP: classical secreted proteins; NCSP: non-classical secreted proteins; *P ≤ 0.05; **P ≤ 0.01; ***: P ≤ 0.001

Table 7. Number of different expression patterns for the different classified proteins in the cotton xylem sap under the LK and NK treatments based on the results presented in Supplemental Table 1. Note: LK: low K; NK: normal K; NLK: being non-detectable in LK; NNK: being non-detectable in NK; SDR: significant down-regulation; SUR: significant up-regulation; NSC: no significant change.

Supplemental Table 1). Many authors have also reported the appearance or disappearance of specific peroxidase isoforms during a particular process or in a particular localization. The diversity of processes that are catalyzed
by peroxidases and their numerous genes suggest the possibility of functional specialization of each isoform 29. Therefore, further research into the function of the proteins that disappeared or appeared in the xylem sap may facilitate an understanding of how cotton responds to potassium deficiency. Additionally, some proteins were identified that have not been previously associated with environmental-stress-related proteins. These proteins were classified into miscellaneous proteins. Research into these proteins and the significant change or absence of uncharacterized proteins between the LK and NK treatments will provide additional information regarding their expression patterns and roles in a plant’s response to environmental stresses and plant root growth.

Proteins in the xylem sap might originate from the secretion of adjacent xylem parenchyma or pericycle cells via the classical (containing a signal peptide) or the non-classical (lacking a signal peptide) secretion pathway 16,24. In our study, 90.31% of the xylem sap proteins were secreted proteins, which was composed of 56.98% classical peptides and 33.33% non-classical peptides; 9.69% were non-secreted proteins, e.g., lactoylglutathione lyase (glyoxalase I) (an enolase). Similarly, enolase has been secreted to the cell wall or extracellular space via immunolocalization even though it lacked a signal peptide 30. Furthermore, glyoxalase 1 has been identified in the cell wall proteome of the maize primary root elongation zone 31 and in a cell wall proteomics study of mature alfalfa stems 32. In addition, the DNA-binding protein histone H4 has been unexpectedly found among secreted proteins 25 and in the cotton xylem sap (Table 7; Supplemental Table 1), although it was predicted as not secreted in the current study (Table 7; Supplemental Table 1).

The non-secreted proteins might result from tracheid development or through direct release after the death of xylem cells, resulting from programmed cell death 33. The presence of histone in the secretome proteins of the pea root cap is regarded as a global leakage of material from disrupted nuclei in dead cells 25. Potassium couldn’t increase histone 4 gene transcription (Fig. 4), so it might obviously promote histone 4 protein translation, cell membrane damage and nuclei disruption, and obviously lead to histone 4 increase in xylem sap. On the other side, it might be a positive response due to innate immunity. Moreover, most cell wall proteins belong to multiprotein...
Figure 4. Coordination of protein expression and each corresponding gene expression in K-deficient cotton root and the control.
families, and proteins in the same family can have different cellular localizations. For example, the glycoside hydrolase family proteins in this study were predicted as classical secreted proteins, non-classical secreted proteins, or non-secreted proteins (Supplement Table 1).

Root growth and development are complex processes, comprising cell proliferation, elongation and differentiation, which involve cell wall extension and remodeling by glycosyltransferases (GTs), glycoside hydrolases (GHs) and expansin-like proteins34. Changing cell wall properties may affect lateral root emergence from the parent root34,35. GTs and GHs are two large superfamilies of carbohydrate-active enzymes. All GTs catalyze the transfer of sugar moieties to acceptor molecules. In contrast, GHs hydrolyze bonds that exist between sugar moieties and other molecules. Within each of these superfamilies, nearly all of the proteins were down-regulated or not detectable under the potassium-deficient conditions, suggesting that the cell wall metabolism was largely inhibited by potassium deficiency.

Significantly, potassium deficiency has been shown to inhibit cotton root elongation and lateral root formation35, which is consistent with the general decrease in GTs, GHs and expansin-like proteins observed in this study (Table 7; Supplemental Table 1). In addition, fasciclin-like arabinogalactan proteins (FLAs) are necessary for normal Arabidopsis root growth37. According to the GO biological process annotation, the xylem cysteine peptidase 1 takes part in developmental programmed cell death and in the regulation of meristem growth, and a peroxidase (A0A061DQ02) takes part in the regulation of meristem growth. These proteins were significantly down-regulated (0.20 fold change (FC) for xylem cysteine peptidase 1; 0.12 FC for A0A061DQ02 and 0.12 FC for FLA 12) or were absent (NLK for FLA 10), further indicating that potassium deficiency inhibited root elongation and branching at the protein level.

Plants cannot escape environmental harm; thus, adaptability and tolerance to biotic and abiotic stresses is very important for plant survival and growth. The resistance of plants to biotic and abiotic stresses can be divided into pre-formed or passive defense and active defense tactics, responding to stresses that circumvent pre-formed barriers38,39. Based on significantly different protein levels seen between the LK and NK treatments, it can be concluded the former was related to the cell wall barrier (proline/hydroproline-rich proteins), antifungal proteins and enzymatic inhibitors, whereas the latter was related to recognition and signaling (Fig. 5).

Hydroxyproline-rich glycoproteins (HRGPs) are important plant cell wall components that are involved in plant disease resistant responses, especially in incompatible plant-pathogen interactions, acting as impenetrable physical barriers against pathogen ingress40. Arabinogalactan proteins (AGPs) are a class of Hyp-rich glycoproteins, and fasciclin-like AGPs (FLAs) constitute a fourth distinct subclass of AGPs41. Lignin is a strengthening polymer that provides not only structural support but also defense against pathogens. Lignin is formed within the plant cell wall matrix by laccase and class III peroxidases. Additionally, peroxidase takes part in the production of phytoalexins42. Thus, the significant decrease or lack of FLAs or AGPs and peroxidases suggests that potassium deficiency decreased the pre-formed mechanical and biochemical resistance of the cotton root cell wall to pathogen attacks.

One group of proteins that has been closely associated with plant defense is PR proteins. Currently, PR proteins are categorized into 17 families according to their properties and functions, including β-1, 3-glucanases, chitinases, thaumatin-like proteins, peroxidases, non-specific lipid transfer proteins, endo-proteases and protease inhibitors43. Chitinases and β-1, 3-glucanases are two important hydrolytic enzymes that are abundant in many plant species. The PR-5 family includes proteins that are related to thaumatin and osmotin, with several members possessing antimicrobial properties44. Non-specific lipid-transfer proteins (LTPs) are small, basic, secreted proteins that modulate a plant’s response to biotic stress45. Among the proteases inhibitors, polygalacturonase-inhibiting proteins (PGIPs) belong to the large superfamily of Leu-rich repeat (LRR) proteins46 and are present in the cell walls of all plants examined to date. These proteins specifically inhibit endo-polygalacturonases (PGs) of fungi but not those of plants or bacteria. The Kunitz trypsin inhibitor exhibits antifungal capabilities and plays an important role in tobacco’s defense response47. Peroxidases were also classified as pathogenesis-related proteins involved in plant defense42. Generally, PR proteins were significantly down-regulated or lacking under the LK treatment, especially class III peroxidases, indicating a weak passive defense mechanism.

If pathogens circumvent the pre-formed defense system that is weakened in the root apoplast by potassium deficiency, more efficient active defense mechanisms might be required. Active defense requires the plant host
to recognize pathogens, signal and activate the related genes to fortify the cell wall, produce phytoalexins, and induce PR proteins.

Given that plant immunity is based on the recognition and constant surveillance of pathogens, the activation of plant defense relies on the recognition of microbial GlcNAc-containing glycans (chitin) that are not inherent to plants themselves; LysM proteins directly or indirectly mediate the recognition of such structures\(^5\). Therefore, plant LysM proteins are involved in defense signaling against fungal attacks. Some FLAs are likely to be attached to the plasma membrane through a glycosylphosphatidylinositol anchor and may interact with receptor-like kinases, such as wall-associated kinases\(^6\). Lectins are the only plant proteins that recognize glycoconjugates (glycoproteins, glycolipids, or polysaccharides) on the surfaces of microorganisms, such as bacteria and fungi. The broad spectrum of the carbohydrate-binding specificity of lectins can be interpreted as the successful recognition of different types of sugar-containing receptors by plant cells\(^7\). Receptor-like protein kinases and NSF-interacting kinase that are localized on the plasma membrane play important roles in biotic stress responses\(^8,9\). Therefore, the significant decrease or lack of these proteins under potassium-deficient conditions might substantially reduce plant recognition, signaling and the subsequent response to environmental stress.

PR proteins and receptor-like kinases are involved in not only biotic but also abiotic stress responses\(^6\). PR-2 and PR-11 are able to adjust their function according to the nature of the stress, e.g., inhibiting their glucanase and chitinase activities during cold stresses and inducing antifreeze activity. Similarly, thiamatin-like proteins have antifreeze capabilities. The three PR-proteins in the apoplastic space have been shown to inhibit the recrystallization of intercellular ice and prevent the formation of intracellular ice\(^10,11\). Individual gene transformation increases plant resistance to abiotic stress. For example, the overexpression of *LTP3* constitutively enhanced Arabidopsis tolerance to freezing stress\(^12\); the constitutive expression of a grape aspartic protease gene in transgenic Arabidopsis confers osmotic stress tolerance\(^13\); and the constitutive expression of a trypsin protease inhibitor confers multiple stress tolerance in transgenic tobacco\(^14\). Further, a particular subset of AtPrx (a peroxidase) genes and their appreciate expressions are required for the cold tolerance\(^15\), indicating that a subset of phylogenetically close peroxidases that decrease or are lacking may be essential for potassium-deficiency tolerance (Fig. 2).

Potassium deficiency generally reduces plant resistance to biotic and abiotic stresses\(^13,16\), due to significantly decreasing or eliminating environmental stress response proteins in cotton seedling xylem sap. Some reports have also shown that potassium deficiency enhances plant resistance to some pathogens\(^5,6\), which is likely due to the significant increase in individual protein isoforms, such as proteinase inhibitors, non-specific lipid-transfer proteins, histones and uncharacterized proteins, under the LK treatment.

It might be possible to enhance plant resistance to environmental stresses using biotechnology to increase the presence of individual genes. However, most plant traits, such as drought and salt tolerance, and insect resistance are controlled by multiple genes. These genes interact via signaling pathways in response to biotic and abiotic stresses\(^17\). In this respect, improving potassium management can also enhance plant resistance to the environment by positively affecting the activation of many genes, which might be applicable over a broader scope than biotechnological methods.

**Methods**

**Cotton seedling culture and potassium deficiency treatment.** The cotton cultivar DP 99B was used in this study. The experiments were conducted in a growth chamber under the following conditions: 30/25 °C, 14/10 h light/dark period, and 450 μmol m\(^{-2}\) s\(^{-1}\) light. The seeds were surface sterilized with 10% H\(_2\)O\(_2\) for thirty minutes, washed with tap water three times, and soaked for 12 h in tap water. The soaked seeds were germinated and emerged in wet sand. Only those seedlings that emerged well were transferred to a culture solution. The solution contained 2.5 mM KCl, 2.5 mM Ca(NO\(_3\))\(_2\), 1 mM MgSO\(_4\), 0.5 mM NH\(_4\)H\(_2\)PO\(_4\), 2 mM NaCl, 2 × 10\(^{-4}\) mM CuSO\(_4\), 1 × 10\(^{-3}\) mM ZnSO\(_4\), 0.1 mM EDTA-FeNa, 2 × 10\(^{-2}\) mM H\(_2\)BO\(_3\), 5 × 10\(^{-6}\) mM (NH\(_4\))\(_6\)Mo\(_7\)O\(_24\), and 1 × 10\(^{-3}\) mM MnSO\(_4\). This transferring point in time was denoted as 0 d after transferring (DAT). Seedlings at 3 DAT were treated with K at normal K (2.5 mM KCl) and deficient K (0.05 mM) levels. Sodium ions were provided using NaCl to the seedlings that were exposed to low-K conditions; the other mineral nutrients remained unchanged. After 7 d of K treatment, the samples were collected.

**Cotton seedling organ sampling and cation content determination.** The seedlings were separated into root, stem including the leaf petiole, and leaf components, oven-dried at 80 °C until a constant dry weight was attained, and weighed. Ions in the dried and ground root, stem and leaf components were extracted with 1 mM HCl for 24 h under room temperature and via rotation (150 rpm). The extracted solution was centrifuged (5000 rpm), and the supernatant liquid was used for ion determination using inductively coupled plasma-optical emission spectrometry (PE-optima 2100 DV, USA)

**Xylem sap collection.** At 7 d of potassium deficiency, xylem sap was collected after cutting the stems approximately 5 cm above the junction of the root and the stem under “root pressure”. After thoroughly washing each rootstock surface with distilled water, they were blotted with filter paper, a latex tube was fitted over the rootstock, and the other end of the latex tube was placed into a plastic tube contained in a foam box filled with ice (supplementary Fig. 1). Xylem sap was collected for 48 h and then kept at −80 °C. The xylem sap of each biological replication was collected from 6–8 plants and pooled for protein content determination and from 12–16 plants for label-free protein quantification.

**Protein preparations.** Xylem sap was thawed and filtered through 0.2 μm cellulose acetate filters. The filtered xylem sap with the same volume for each biological replication of different K treatments was used to acquire the concentrated proteins using a 3 KD ultra-centrifugal filter device (Amicon Ultra-4, Merk Millipore,
Darmstadt, Germany). The concentrated proteins were used for determination and for protein digestion after being precipitated using acetone.

**Determination of antioxidant enzyme activity and their gel activity analysis using modified SDS-PAGE.** SOD (EC 1.15.1.1) activity was measured using the NBT photochemical method. One unit of SOD activity was defined as the amount of enzyme required for the 50% inhibition of the rate of NBT reduction at 560 nm, and SOD activity was expressed as unit ml⁻¹ xylem sap or unit μg⁻¹ protein. GPX (EC 1.11.11.7) activity was determined using guaiacol at 470 nm. The 3-mL reaction mixture contained 100 mM potassium phosphate (pH 6.5), 16 mM guaiacol, 10 mM H₂O₂ and 3 μL concentrated protein solution. The reaction was initiated upon the addition of concentrated protein solution.

Modified SDS–PAGE was used to separate peroxidase isoforms and SOD isoforms by molecular weight using the prothentic haem group. The final concentration of SOD was 0.1% (w/v) in all solutions and gels. Samples were diluted in loading buffer to final concentrations of 62.5 mM TRIS-HCl, 0.1% (w/v) SDS, 10% (w/v) glycerol, and 0.002% (w/v) bromophenol blue without reducing compounds and loaded onto the gels without heating. After completion of electrophoresis, for proteins, the gel was stained by coomassie brilliant blue. For SOD activity, the gel was incubated in a solution containing 2.45 mmol/L NBT for 25 min, followed by incubation in 50 mmol/L potassium phosphate buffer (pH 7.8) containing 28 μmol/L riboflavin and 28 mmol/L TEMED under dark conditions. Expression of SOD was achieved by light exposure for 10 to 20 min at room temperature. For GPX activity, the gel was stained with 16 mM guaiacol used as a substrate for peroxidase reaction and 10 mM H₂O₂ in 100 Na-acetate buffer pH 5.0.

**Peptide preparations.** Protein digestion was performed according to the FASP (filter-aided sample preparation) procedure described by Wiśniewski et al.39 Briefly, each protein pellet was solubilized in 200 μL SDT buffer (4% SDS, 10 mM DTT, and 150 mM Tris-HCl, pH 8.0) in a boiling water bath for 30 min, amended with DTT to a final concentration of 100 mM, and bathed at 100°C for 5 min. The solution was then transferred to a 10 kD ultra-centrifugal filter device, amended with 200 μL of UA buffer (8 M Urea, and 150 mM Tris–HCl, pH 8.0), and centrifugally ultra-filtered at 14000 g for 15 min to remove the detergent, DTT and other low-molecular-weight components. Then, the filter device was amended with 100 μL of iodoacetamide (IAA) (50 mM IAA in UA), vibrated (660 rpm, 1 min), placed in darkness at room temperature and centrifuged at 1400 g for 10 min. The tube was amended with 100 μL UA buffer and centrifuged at 1400 g for 10 min, which was repeated twice. The same process was performed with 100 μL 25 mM NH₄HCO₃. In the above processes, the liquid filtrate was discarded each time. Finally, the suspended protein was initially digested with 2 μg trypsin (Promega) in 40 μL 25 mM NH₄HCO₃ with vibration (600 rpm, 1 min) and subsequently held at 37°C for 18 h; the resulting peptides were collected as the filtrate.

**UPLC-MS/MS.** Peptide mixtures were analyzed by on-line nanoflow liquid chromatography using the EASY-nLC 1000 system (Thermo Finnigan) with a trap column (EASY column SC001 traps; 150 μm × 20 mm (RP-C18)) and analysis column (EASY column SC200; 150 μm × 100 mm (RP-C18)). Each sample was auto-sampled into the trap column and separated by the analysis column at a flow rate of 400 nL/min. The analysis column was balanced with 100% mobile phase A (water solution with 0.1% formic acid and 2% acetonitrile). Peptides were eluted with a linear gradient from 0–45% mobile phase B (water solution with 0.1% formic acid and 84% acetonitrile) for 100 min and 45–100% B for 8 min and maintained at 100% for 12 min. The eluate was electro-sprayed into a Q-Exactive Orbitrap Mass (Thermo Finnigan) for 120 min. The Q-Exactive was operated with one full precursor scan scope (m/z 300–1800) (MSⁿ scan) and in a HCD top 10 mode (MS²⁺ scan). The resolution was 70,000 for the full scan and 17,500 for the fragments (both specified at an m/z of 200). The exclusion time was 90 sec. Raw files were processed using MaxQuant version 1.3.9.3 (http://www.maxquant.org/) with the iBAQ and match between runs (match time window 2 min) options. For protein identification, the MS/MS spectra were automatically searched by MaxQuant against the target/reverse UniProt Eudicotyledons database (FASTA-formatted protein sequence database). The identified proteins were further statistically and bioinformatically analyzed using Perseus version 1.3.04. The fixed modification was carbamidomethyl (C). The variable modifications were oxidation (M) and acetyl (protein N-term). The initial mass tolerances for the full scans were 6 ppm and 20 ppm for MS/MS. Two missed cleavages were allowed. The peptide and protein false discovery rates (FDR) were both set to 0.01.

**Proteins quantification.** The iBAQ (intensity-based absolute quantification) option was used in MaxQuant to calculate the approximate share of each protein in the total proteome based on the sum of the peak intensities40. If the iBAQ of a protein was detected two or three times from three biological replicates in the NK and LK treatments, the protein was quantified and compared between the two treatments. If the iBAQ of a protein was detected two or three times from three biological replicates in NK or LK (i.e., only one of the treatments), the protein was termed as being non-detected in LK or non-detected in NK, respectively.

**GO annotations and locations of identified proteins.** The gene ontology (GO) annotations in terms of cellular components, molecular functions and biological processes for the identified proteins were obtained from http://www.uniprot.org. The theoretical molecular weights (MWs) and isoelectric points (pIs) of the proteins were collected from http://www.ebi.ac.uk/Tools/seqstats/emboss_peptides/. The proteins were predicted for secretion with a signal peptide using SignalP (www.cbs.dtu.dk/services/SignalP/) and without a signal peptide using SecretomeP (www.cbs.dtu.dk/services/SecretomeP). In addition, the non-secreted proteins that were predicted by SignalP and SecretomeP were predicted by TargetP (www.cbs.dtu.dk/services/TargetP) for their locations.
Phylogenetic analysis of peroxidase proteins. A phylogenetic tree was constructed using neighbor joining (NJ) approaches, among which phylogenetic analyses were conducted using MEGA version 5.1 with the following parameters: model (p-distance), bootstrap (1,000 replicates) and gap/missing data (pairwise deletion).

Calculation of gene expression levels. To obtain comprehensive transcription profile of proteins listed in Table 6 for K-deficient cotton root, we use the Illumina HiSeq2000 to perform high-throughput RNA-seq data of K-deficient root and K-efficient root. In total, 8.99 Gb of raw RNA-seq data were generated (BGI-Tech., China).

RNA-seq reads were mapped to the cotton genotype TM-1 genome using Tophat (Version 2.0.8). To measure the gene expression level in LK and NK root tissues, we calculated the expression of each gene using FPKM (fragments per Kilobase of exon model per Million mapped reads) with Cufflinks (Version2.1.1) (http://cufflinks.cbcb.umd.edu/).

We analyzed the gene (corresponding to proteins listed in Table 6) expression changes of K-deficient cotton root, compared with K-efficient root, and present a heatmap for the coordination of gene transcription and protein expression by using software MultiExperiment Viewer (MeV).

Statistics. Experiments for effects of potassium deficiency on cotton seeding growth, mineral nutrient contents, xylem sap volume, proteins contents and pH of xylem sap were repeated three times with similar results. Qualification and quantification of proteins in cotton xylem sap of one of three experiments was done. Treatment means were compared using t-test.

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Author Contributions
Z.Y.Z and Q.L.W. designed the study and wrote the paper; M.N.C. analyzed xylem sap proteins quantification, classification and evolutionary relationships of peroxidases; Z.Y.Z., S.E.W., J.J.B., J.X.T. and F.L. cultivated cotton seedlings, collected xylem sap and determined dry seedlings weight, minerals contents, and xylem sap pH and protein contents; B.H.Z. improved the paper.

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