RESEARCH PAPER

Mechanism of phytohormone involvement in feedback regulation of cotton leaf senescence induced by potassium deficiency

Ye Wang¹, Bo Li², Mingwei Du¹, A. Egrinya Eneji³, Baomin Wang¹, Liusheng Duan¹, Zhaohu Li¹ and Xiaoli Tian¹,*

¹ State Key Laboratory of Plant Physiology and Biochemistry, Key Laboratory of Crop Cultivation and Farming System, Center of Crop Chemical Control, China Agricultural University, Beijing 100193, China
² ShanDong Kingenta Ecological Engineering Co., Ltd, China
³ Department of Soil Science, Faculty of Agriculture, Forestry and Wildlife Resources Management, University of Calabar, Nigeria

* To whom correspondence should be addressed. E-mail: tianxl@cau.edu.cn

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Abstract

To elucidate the phytohormonal basis of the feedback regulation of leaf senescence induced by potassium (K) deficiency in cotton (Gossypium hirsutum L.), two cultivars contrasting in sensitivity to K deficiency were self- and reciprocally grafted hypocotyl-to-hypocotyl, using standard grafting (one scion grafted onto one rootstock), Y grafting (two scions grafted onto one rootstock), and inverted Y grafting (one scion grafted onto two rootstocks) at the seedling stage. K deficiency (0.03 mM for standard and Y grafting, and 0.01 mM for inverted Y grafting) increased the root abscisic acid (ABA) concentration by 1.6- to 3.1-fold and xylem ABA delivery rates by 1.8- to 4.6-fold. The K deficiency also decreased the delivery rates of xylem cytokinins [CKs; including the zeatin riboside (ZR) and isopentenyl adenosine (iPA) type] by 29–65% and leaf CK concentration by 16–57%. The leaf ABA concentration and xylem ABA deliveries were consistently greater in CCRI41 (more sensitive to K deficiency) than in SCRC22 (less sensitive to K deficiency) scions under K deficiency, and ZR- and iPA-type levels were consistently lower in the former than in the latter, irrespective of rootstock cultivar or grafting type, indicating that cotton shoot influences the levels of ABA and CKs in leaves and xylem sap. Because the scions had little influence on phytohormone levels in the roots (rootstocks) of all three types of grafts and rootstock xylem sap (collected below the graft union) of Y and inverted Y grafts, it appears that the site for basipetal feedback signal(s) involved in the regulation of xylem phytohormones is the hypocotyl of cotton seedlings. Also, the target of this feedback signal(s) is more likely to be the changes in xylem phytohormones within tissues of the hypocotyl rather than the export of phytohormones from the roots.

Key words: Abscisic acid, cotton, cytokinins, feedback regulation, potassium deficiency.

Introduction

The wide adoption of Bt cotton cultivars, noted for greater susceptibility to potassium (K) deficiency (Zhang et al., 2007; Yang et al., 2011), and inadequate input of K fertilizer has brought about an increasing occurrence of premature senescence in Chinese cotton-producing areas (Dong et al., 2006; Tian et al., 2008), characterized by early chlorophyll degradation and reduced photosynthesis in mature leaves (Bednarz and Oosterhuis, 1995; Zhao et al., 2001) during flowering and boll development. The widespread K deficiency caused by the use of higher yielding and faster fruiting cotton cultivars across the Cotton Belt of the USA was also reported about two decades ago (Oosterhuis et al., 1995).
It is generally considered that cytokinins (CKs) and abscisic acid (ABA) are two major phytohormones involved in the initiation and progression of plant senescence. The results of measurement of endogenous CK levels before and during senescence (van Staden et al., 1988; Singh et al., 1992; Gan and Amasino, 1995), external application of CKs (van Staden et al., 1988; Zavaleta-Mancera et al., 1999), and manipulation of endogenous production of CKs in transgenic plants (Gan and Amasino, 1995; Rivero et al., 2007; Ghanem et al., 2011a) showed that CKs can inhibit leaf senescence. The gain-of-function analyses also indicated that CK receptors AHK2 and AHK3 participate in regulating the onset of leaf senescence in Arabidopsis (Kim et al., 2006; Riefler et al., 2006). In contrast to CKs, the ABA level increases in senescing leaves (Gepstein and Thimann, 1980; Samet and Sinclair, 1980), and genes coding for proteins involved in ABA synthesis and signalling are up-regulated during leaf senescence (Buchanan-Wollaston et al., 2005; van der Graaff et al., 2006; Jukanti et al., 2008). Furthermore, it has been known that exogenous application of ABA promotes leaf abscission and senescence (Zeevaart and Creelman, 1988), and exogenously applied ABA induces the expression of several SAGs (senescence-associated genes; Weaver et al., 1998), which is consistent with its promotion of leaf senescence.

Nutrient deprivation (1% or 10% full-strength nutrient solution) not only decreased the levels of CKs in shoot and root (Kuiper et al., 1989; Vysotskaya et al., 2009), but also caused an accumulation of ABA in the shoot (Vysotskaya et al., 2009). It is well known that CK levels in roots, shoots, and xylem sap are reduced in nitrogen-starved plants (Takei et al., 2001; Dodd et al., 2004). With respect to K, although increased ABA levels have been measured in grains and flag leaves of wheat (Triticum aestivum) (Haeder and Beringer, 1981), roots, xylem, and phloem sap of castor bean (Ricinus communis L.) (Jeschke et al., 1997; Peuke et al., 2002), and leaves of Arabidopsis (Kim et al., 2009), the physiological consequences of this increase in ABA are less well known. One report (Salama and Wareing, 1979) showed that K deficiency also caused a marked decrease in CK levels in both roots and leaves.

A previous grafting study determined the roles of shoot and root in the regulation of premature senescence induced by K deficiency in cotton (Li et al., 2012). The results showed that the effect of rootstocks on leaf senescence was significant in some cases, but the scion cultivars explained a higher percentage of variation within grafting treatments. It was speculated that the shoot-to-root feedback signal(s) that mediates xylem phytohormone delivery was involved in the shoot regulation of premature senescence of cotton (Li et al., 2012).

Several studies have shown that the root system dominates the root export of CKs via the xylem (Sitton et al., 1967; McKenzie et al., 1998; Dong et al., 2008; Albacete et al., 2009, 2010; Ghanem et al., 2011b). However, results from studies on grafted pea branching mutants and Arabidopsis branching mutants suggested a feedback regulation of xylem sap CKs by some long-distance signals that move from shoot to root (Beveridge et al., 1997; Foo et al., 2007). Furthermore, the interaction of root and shoot in terms of xylem phytohormone delivery may exist when considering the recirculation of phytohormones between xylem and phloem (Dodd, 2005).

Although premature senescence is a typical symptom of K deficiency in cotton (Bednarz and Oosterhuis, 1995; Wright, 1999; Zhao et al., 2001), there has not been much attention paid to the role of endogenous hormones in the leaf senescence induced by K deficiency. Therefore, the objectives of this study were to (i) examine the effects of K deficiency on the endogenous ABA, zeatin riboside (ZR) + zeatin (Z), and isopentenyl adenosine (iPA) + isopentenyladenine (iP) levels in roots, leaves, and xylem sap of cotton seedlings; (ii) determine whether there is a linkage between leaf senescence induced by K deficiency and changes in endogenous CK and ABA levels in leaves; and (iii) test the hypothesis that the leaf and xylem CK and ABA levels are dependent on feedback signal(s) derived from the shoot by using a grafting study as in previous work (Li et al., 2012). The results should aid our understanding of mechanisms of phytohormone involvement in the feedback regulation of leaf senescence induced by K deficiency, and facilitate the development of approaches to managing this problem.

Materials and methods

Plant material

Two cotton cultivars contrasting in sensitivity to K deficiency were used in the present study: CCR141, developed by the Cotton Research Institute, Chinese Academy of Agricultural Sciences, which is more likely to senesce under K deficiency, and SCRC22, developed by the Cotton Research Center, Shandong Academy of Agricultural Sciences, which shows relatively late senescence under the same condition.

Growth conditions

The experiment was performed in a growth chamber with 12h light/12h dark at 30±2/22±2 °C, 70–80% humidity, and 600 µmol m–2 s–1 photosynthetically active radiation. Seeds were surface-sterilized by soaking in 9% H2O2 for 30min, rinsed with tap water, and then germinated in K-free sand medium for 4 d in the dark. After germination, uniform seedlings were cultured hydroponically by transferring them to 16 cm×13 cm×16 cm plastic pots filled with 2.2 litres of half-strength modified Hoagland’s solution. The constituents of the solution were (mM) 2.5 Ca (NO3)2, 1 MgSO4, 0.5 (NH4)2HPO4, 2×10–4 CuSO4, 1×10–5 ZnSO4, 0.1 Fe Na EDTA, 2×10–5 H2BO3, 5×10–6 (NH4)2MoO4, and 1×10–3 MnSO4. The concentration of K in the form of potassium sulphate (K2SO4) in the solution was 0.1 mM (mild K deficiency) before grafting and during graft recovery to ensure either a higher survival rate of grafts or faster occurrence of leaf senescence induced by severe K deficiency (0.03 mM or 0.01 mM) after graft establishment.

Grafting

Standard, Y, and inverted Y grafting of cotton seedlings were performed hypocotyl-to-hypocotyl at the cotyledonary or one-leaf stage as described previously (Li et al., 2012). For each type of grafting, two cultivars were self- and reciprocally grafted; standard grafts are denoted as scion/rootstock, and ‘Y’ and ‘inverted Y’ grafts are denoted as (scion+scion)/rootstock and scion/rootstock+rootstock, respectively. After establishment under high humidity and low light (80 µmol m–2 s–1), surviving grafts were transferred to severe K-deficient (0.03 mM for standard and Y grafts, and 0.01 mM for inverted Y grafts because of its two rootstocks providing nutrients for only one scion) solutions to induce premature senescence, with a 2.5 mM (K-sufficient) medium as control. One week after establishment, the cotyledons and axillary bud from cotyledonary nodes of rootstock (standard and Y grafts) were removed.
Sampling of xylem sap
At the 8–9 leaf stage (~32 d after severe K deficiency treatment as above), xylem sap was collected basically according to Noodén et al. (1990). Ten grafts per treatment were decapitated 5–10 mm above the graft union to collect scion xylem sap, and another 10 grafts per treatment were cut below the graft union (~5 mm away) to collect rootstock xylem sap. The cut surface was wiped with distilled water to remove disrupted cells and residual cell elements, then a flexible silicon tube (length 15 mm, internal diameter 2 mm) was placed ~5–10 mm over the stump and tied tightly in place. The collection period started at 10:00 h and lasted for 24 h in the dark each time. After collection, the sap volume was quickly determined to calculate the sap flow rate over the collection period, and then the sap was freeze-dried (SIM FD5-6, LA, USA) and stored in the dark at –40 °C. The xylem ABA and CK delivery rates were calculated by multiplying phytohormone concentrations by the xylem sap flow rates.

Extraction and purification of phytohormone in leaves and roots
The fourth main-stem leaf from the apex and the whole roots were harvested before and after xylem sap collection to determine CKs and ABA. About 0.5 g of fresh samples was extracted and homogenized in 2 ml of 80% methanol (containing 40 mg l−1 butylated hydroxytoluene as an antioxidant). The extract was incubated at 4 °C for 48 h, and then centrifuged at 4000rpm for 15 min at 4 °C. The supernatant was passed through C18 Sep-Pak cartridges (Waters Corp., Millford, MA, USA), and the phytohormone fraction was eluted with 10 ml of 100% (v/v) methanol and then 10 ml of ether. The eluate was dried down by pure N2 at 20 °C, and then stored at –40 °C.

Quantification of ABA and CKs by enzyme-linked immunosorbent assay (ELISA)
Freeze-dried xylem sap and N2-dried extracts of leaf and root samples were dissolved in 2.0 ml of phosphate-buffered saline (PBS) containing 0.1% (v/v) Tween-20 and 0.1% (w/v) gelatin (pH 7.5) to quantify free ABA, ZR, and iPA by ELISA following the protocol described in Zhao et al. (2006). The mouse monoclonal antigen and antibodies against free ABA, ZR, and iPA were produced at the Center of Crop Chemical Control, China Agricultural University, China, according to Weiler et al. (1981). As the anti-ZR antibody also detects Z, the CKs quantified by this antibody are described as ZR-type CKs. Similarly, the anti-iPA antibody detects iP and iPR, hence the CKs quantified by this antibody are described as iPA-type CKs. Calculations of the ELISA data were performed as described in Weiler et al. (1981). The recovery percentage obtained by using internal standards during extraction and analysis was all >90%.

Leaf photosynthesis measurement
In a separate experiment with four replicates and four plants for each replicate (Li et al., 2012), the photosynthesis rate (Pn) of the fourth main-stem leaf from the apex (functional leaf) was measured by a Li-6400 portable photosynthesis system (LI-COR, Lincoln, NE, USA) with 1000 µmol m−2 s−1 quantum flux and 500 µmol mol−1 CO2 concentration.

Data analysis
Three replicates were used for each grafting treatment, and each replicate consisted of 10 plants for each rootstock or scion xylem sap samples and 20 plants for leaf and root samples. A similar trend of results was found in three independent repeat experiments; thus data were pooled across repeats. Analysis of variance (ANOVA) was performed using SAS statistical software (V8, SAS Institute Inc., Cary, NC, USA) to determine the significance of the effects of K supply, rootstock, and scion, and their interaction under K deficiency. Treatment means were compared using Duncan’s multiple range test.

Fig. 1. Effect of K deficiency on ABA levels of cotton standard graft (scion/rootstock) at the 8–9 leaf stage. Grafting was performed hypocotyl-to-hypocotyl at the 1-leaf stage of the rootstock and the cotyledonary stage of the scion. Grafts were maintained in nutrient solution with 0.1 mM K during establishment, and transferred to solutions with either 2.5 mM (a) or 0.03 mM K (b) after establishment. The ABA concentrations (ng g−1 FW) in roots (R) and the youngest fully expanded leaf (L), and xylem ABA delivery rates (ng plant−1 24 h−1) below (B) and above the graft union (A) were determined. For each K level, means of the same sampling part (i.e. roots, leaf, and xylem sap collected both below and above the graft union) followed by the same letter are not significantly different according to Duncan’s multiple range test, P < 0.05, n=4.
Results

Spatial pattern of ABA and CKs in cotton seedlings

As shown in Figs 1, 3, and 5, the ABA concentrations in cotton leaves were much higher than those in roots. The iP-type concentrations in roots and leaves were higher than those of the ZR-type, whereas the xylem iP-type delivery rate was much lower than that of the ZR-type (Figs 2, 4, 6), indicating that the ZR-type is the dominant type of CKs in cotton xylem sap, as in other plants (Singh et al., 1992; Beveridge et al., 1997; Foo et al., 2007).

There were no significant differences in the xylem ABA and CK delivery rates (the product of phytohormone concentration in the xylem sap and sap flow rate) between rootstock (collected below the graft union) and scion (collected above the graft union) within a standard graft (Figs 1, 2), implying that the graft union itself had little influence on phytohormone delivery as previously demonstrated (Holbrook et al., 2002; Dodd et al., 2008). In addition, the sum of the xylem phytohormone levels of the two scions was more than that of the rootstock within a Y graft (Figs 3, 4), and the xylem phytohormone level in the scion was less than that of the sum of the two rootstocks within an inverted Y graft (Figs 5, 6), suggesting that the scion xylem phytohormone deliveries were independent of the xylem of the rootstocks.

ABA and CK levels in standard grafts

ABA

Under K sufficiency, there were no significant differences between SCRC22 and CCRI41 in either root (rootstocks) ABA concentrations or leaf (scion) ABA concentrations (Fig. 1a). Also, SCRC22 had similar xylem ABA delivery rates to CCRI41 both in rootstocks and in scions (Fig. 1a).

When submitted to K deficiency, the root ABA concentrations and xylem ABA delivery rates across grafts significantly ($P < 0.001$) increased by 1.6- and 4.6-fold, respectively, whereas the leaf ABA concentrations changed little (Fig. 1b; Table 1). Compared with SCRC22 self-grafts, CCRI41 self-grafts had 88, 90, and 28% greater ABA levels in roots, leaves, and xylem sap, respectively. In addition, it was observed that although the SCRC22 rootstock could reduce the ABA levels in leaves and xylem sap of CCRI41 scions compared with CCRI41 self-grafts, the corresponding values were greater than those of SCRC22 self-grafts. Similarly, the CCRI41 rootstock had a tendency to enhance the ABA levels in leaves and xylem sap of SCRC22 scions compared with SCRC22 self-grafts, but the corresponding values were lower than those of CCRI41 self-grafts (Fig. 1b). These results suggest a feedback regulation of leaf ABA concentrations and xylem ABA delivery rates by scion cultivars. Furthermore, the scion did not affect the rootstock in terms of root ABA concentrations under K deficiency (Fig. 1b; Table 1).

CKs

Under K sufficiency, the ZR- and iP-type concentrations in roots and leaves of SCRC22 tended to be higher than those of CCRI41, but the differences were not significant in most cases. In addition, the xylem ZR-type delivery rates in SCRS22 scions were significantly greater than those in CCRI41 scions regardless of rootstock cultivars. However, there were no significant variations in the xylem iP-type delivery rates between scions of the two cultivars.

When exposed to K deficiency, the roots showed 73% ($P < 0.001$) more ZR-type concentrations across grafts compared with K sufficiency, and a slight but significant ($P = 0.009$) increase occurred in iP-type concentrations (Fig. 2b; Table 1). Nonetheless, the ZR-type levels in leaves and xylem sap decreased by 32% and 29% ($P < 0.001$), and the iP-type levels decreased 48% and 63% ($P < 0.001$), respectively. The ZR- and iP-type levels in leaves and xylem sap of reciprocal grafts were altered insignificantly compared with self-grafts with the same scions as reciprocal grafts, suggesting a feedback regulation by scion cultivars (Fig. 2b). The same cultivar rootstock had similar root ZR- or iP-type concentrations regardless of scion cultivar, indicating little influence of scion on rootstock. Considering genotypic variations, SCRC22 rootstocks across self- and reciprocal grafts showed 26% and 87% greater ZR- and iP-type concentrations in roots (Fig. 2b). Also, SCRC22 scions showed 61% and 60% greater ZR- and iP-type concentrations in leaves, and 26% and 42% greater ZR- and iP-type delivery rates in xylem sap (averaged across above and below the graft union) than CCRI41 scions.

ABA and CK levels in Y grafts

ABA

Under K sufficiency, although there were no typical symptoms of premature senescence, genotypic variations in root and xylem ABA levels were observed between the two cultivars (Fig. 3a). Compared with SCRC22, CCRI41 rootstocks had greater ABA levels not only in roots but also in xylem sap (collected below the graft union) regardless of scion cultivars. Also, the xylem ABA delivery rates in CCRI41 scions were 93% significantly greater than those of SCRC22 scions regardless of rootstock cultivars. There were no significant differences in the leaf ABA concentrations between CCRI41 and SCRC22 scions.

Under K deficiency, the ABA levels significantly increased by 3.1-fold in roots, 1.9-fold in rootstock xylem sap, and 1.6-fold in scion xylem sap compared with K sufficiency (Fig. 3b; Table 1). However, there was no significant alteration in leaf ABA concentrations. CCRI41 rootstocks showed 86% and 32% greater ABA levels in roots and xylem sap than SCRC22 rootstocks; and its scions had consistently greater ABA levels in leaves and xylem sap than SCRC22 scions, even if they were grafted together onto the same rootstock, clearly suggesting a feedback regulation by scion cultivar. The scions did not affect root and xylem ABA levels in rootstocks (Fig. 3b).

CKs

Under K sufficiency, there were no significant differences in root ZR- and iP-type concentrations between SCRC22 and CCRI41 rootstocks. However, the xylem ZR- and iP-type delivery rates of SCRC22 rootstocks were much greater than those of CCRI41 rootstocks in most cases, regardless of scion cultivars. Similarly, SCRC22 scions showed greater xylem ZR- and iP-type levels than CCRI41 scions, even if they were grafted together onto
the same rootstock (Fig. 4a). No significant differences in leaf ZR- and iPA-type concentrations between the two cultivars were observed.

When subjected to K deficiency, the ZR-type levels in roots across grafts increased by 58% ($P < 0.001$) compared with K sufficiency, and iPA-type levels changed slightly but significantly (Fig. 4b; Table 1). However, the ZR-type levels in xylem sap of rootstocks, xylem sap of scions, and leaves decreased by 39, 36, and 41% ($P < 0.001$); and the iPA-type levels decreased by 38, 43 ($P < 0.001$), and 16% ($P = 0.004$), respectively. Compared

### Table 1. Summary of analysis of variance of ABA, ZR-, and iPA-type levels in the cotton grafting experiment

$P$-values are presented for each main effect or interaction.

| Effect or interaction | Root | Xylem sap below the graft union | Xylem sap above the graft union | Leaf |
|-----------------------|------|--------------------------------|---------------------------------|------|
| **Standard grafting** |      |                                |                                 |      |
| ABA                   | K$^a$| <0.001                         | <0.001                          | 0.061|
|                       | S-scion$^b$ | 0.452                         | 0.046                           | 0.027|
|                       | S-rootstock$^c$ | 0.096                         | 0.129                           | 0.881|
|                       | S-scion×rootstock$^d$ | 0.541                         | 0.276                           | 0.591|
|                       | D-scion$^e$ | 0.168                         | 0.067                           | 0.002|
|                       | D-rootstock$^f$ | <0.001                       | 0.033                           | 0.057|
|                       | D-scion×rootstock$^g$ | 0.315                         | 0.756                           | 0.825|
| ZR-type              | K    | <0.001                         | <0.001                          | <0.001|
|                       | S-scion | 0.097                         | 0.003                           | <0.001|
|                       | S-rootstock | 0.002                         | 0.129                           | 0.322|
|                       | S-scion×rootstock | 0.295                         | 0.085                           | 0.651|
|                       | D-scion | 0.024                         | 0.031                           | <0.001|
|                       | D-rootstock | <0.001                       | 0.051                           | 0.040|
|                       | D-scion×rootstock | 0.734                         | 0.984                           | 0.813|
| iPA-type             | K    | 0.009                          | <0.001                          | <0.001|
|                       | S-scion | 0.166                         | 0.613                           | 0.005|
|                       | S-rootstock | 0.262                         | 0.574                           | 0.864|
|                       | S-scion×rootstock | 0.719                         | 0.711                           | 0.320|
|                       | D-scion | 0.070                         | <0.001                          | <0.001|
|                       | D-rootstock | <0.001                       | 0.094                           | 0.043|
|                       | D-scion×rootstock | 0.286                         | 0.923                           | 0.621|
| **Y grafting**       |      |                                |                                 |      |
| ABA                   | K    | <0.001                         | <0.001                          | 0.546|
|                       | S-rootstock | <0.001                       | 0.374                           | 0.770|
|                       | D-rootstock | <0.001                       | 0.850                           | 0.281|
| ZR-type              | K    | <0.001                         | <0.001                          | <0.001|
|                       | S-rootstock | 0.015                         | <0.001                          | 0.662|
|                       | D-rootstock | <0.001                       | 0.657                           | 0.097|
| iPA-type             | K    | <0.001                         | <0.001                          | 0.004|
|                       | S-rootstock | 0.084                         | 0.001                           | 0.251|
|                       | D-rootstock | <0.001                       | 0.159                           | 0.248|
| **Inverted Y grafting** |      |                                |                                 |      |
| ABA                   | K    | <0.001                         | <0.001                          | 0.308|
|                       | S-scion | 0.118                         | 0.089                           | 0.021|
|                       | D-scion | 0.279                         | 0.681                           | <0.001|
| ZR-type              | K    | <0.001                         | <0.001                          | <0.001|
|                       | S-scion | 0.080                         | 0.081                           | 0.001|
|                       | D-scion | 0.017                         | <0.001                          | <0.001|
| iPA-type             | K    | <0.001                         | <0.001                          | <0.001|
|                       | S-scion | 0.100                         | 0.002                           | <0.001|
|                       | D-scion | 0.742                         | <0.001                          | <0.001|

$^a$ K deficiency (0.03 mM for standard and Y grafts, and 0.01 mM for inverted Y grafts) relative to control (2.5 mM) was imposed on cotton grafts after establishment.

$^b$ Scion effect under K sufficiency (S-scion).

$^c$ Rootstock effect under K sufficiency (S-rootstock).

$^d$ Scion×rootstock under K sufficiency (S-scion×rootstock).

$^e$ Scion effect under K deficiency (D-scion).

$^f$ Rootstock effect under K deficiency (D-rootstock).

$^g$ Scion×rootstock under K deficiency (D-scion×rootstock).
with CCR141 self-graft, the rootstock of the SCRC22 self-graft had 37% and 55% greater ZR-type, and 37% and 43% greater iPA-type levels, in roots and xylem sap, respectively (Fig. 4b). When one of two scions of a self-graft was replaced by the other cultivar, the ZR- and iPA-type levels in both SCRC22 or CCR141 rootstocks changed little. SCRC22 scions had more ZR- and iPA-type CKs in leaves and xylem sap than CCR141 scions, even if they were grafted together onto the same rootstock (insignificant for the ZR type; Fig. 4b). The mean values of leaf ZR- and iPA-type concentrations and xylem ZR- and iPA-type delivery rates across SCRC22 scions were 72% and 32%, and 41% and 60% more, respectively, than those across CCR141 scions.

**ABA and CK levels in inverted Y grafts**

**ABA**
Under K sufficiency, there were no significant differences in root and xylem ABA levels between CCR141 and SCRC22 rootstocks, and in xylem ABA delivery rates between CCR141 and SCRC22 scions. Nevertheless, CCR141 scions showed greater leaf ABA concentrations than SCRC22 scions regardless of rootstock (Fig. 5a).

Under K deficiency, the ABA levels in roots and xylem sap across rootstocks strongly increased by 2.3- and 1.9-fold ($P < 0.001$) compared with K sufficiency; and those in xylem sap of the rootstock.
sap across scions increased by 78% \((P < 0.001)\); no significant changes were observed in leaf ABA concentrations (Fig. 5b; Table 1). CCRI41 rootstocks showed significantly greater ABA levels in roots and xylem sap than SCRC22 rootstocks whether in self- or reciprocal grafts (except xylem ABA delivery rates in reciprocal grafts). In addition, the ABA levels in leaves and xylem sap of CCRI41 scions were more than those of SCRC22 scions, even if they were separately grafted onto the same combination of rootstocks, displaying the characteristic of feedback regulation. The mean values of ABA in leaves and xylem sap of CCRI41 scions were 53% and 31% more than those of SCRC22 scions.

CKs
Under K sufficiency, SCRC22 rootstocks had greater ZR- and iPA-type levels in roots and xylem sap than CCRI41 rootstocks, but not all differences were significant (Fig. 6a). Similarly, the xylem ZR- and iPA-type delivery rates in SCRC22 scions tended to be higher than those in CCRI41 scions. However, the leaf ZR- and iPA-type concentrations of SCRC22 scions were equivalent to those of CCRI41 scions.

Under K deficiency, the ZR- and iPA-type levels in roots across grafts increased by 90% and 47% \((P < 0.001)\) but decreased by 33% and 63% \((P < 0.001)\) in rootstock xylem sap, 42% and 73% \((P < 0.001)\) in scion xylem sap, and 45% and 57% \((P < 0.001)\) in leaves (Fig. 6b). SCRC22 rootstocks had more ZR- and iPA-type CKs in roots and xylem sap than CCRI41 rootstocks, even if they were grafted together with the same scion (Fig. 6b). In addition, SCRC22 scions showed more ZR- and iPA-type CKs in leaves and xylem sap than CCRI41 scions, even if they were separately grafted onto the same combination of rootstocks (Fig. 6b), suggesting a feedback regulation by shoot cultivars. The mean ZR- and iPA-type levels in leaves and xylem sap of SCRC22 scions were 64% and 55% (leaves), and 1.2-fold and 61% (xylem sap) more than those of CCRI41 scions.

Discussion

Potassium deficiency increased ABA levels in roots and xylem sap but decreased CK levels in xylem sap and leaves of cotton seedlings

It was observed that the flow rates of xylem sap under K deficiency were lower than those under K sufficiency in the present study (data not shown) since K deficiency decreases root hydraulic conductance (Cabanero and Carvajal, 2007), which may result in the overestimation of the xylem phytomolecule concentration. Considering the observed decrease in xylem CK delivery rates (the product of xylem CK concentration and sap flow rate) was used to compare the effects of different K supplies on xylem CK levels.

In the present study, K deficiency \((0.01 \text{ mM} \text{ or } 0.03 \text{ mM})\) increased root ABA concentrations by 1.6- to 3.1-fold, and xylem ABA flux by 1.8- to 4.6-fold across grafting types and cultivars, as compared with adequate K supply \((2.5 \text{ mM})\). These results agreed with those of Peuke et al. (2002) showing that low K supply increased the deposition of ABA in the roots \((1.9\text{-fold})\), and root-to-shoot ABA signal in the xylem \((4.6\text{-fold})\). However, there was no significant change in leaf ABA concentrations across cultivars regardless of grafting type under K deficiency (Table 1), possibly due to the high degradation of xylem-sourced ABA in leaves (Zhang et al., 1997; Holbrook et al., 2002; Peuke et al., 2002).
The responses of ZR- and iPA-type CKs to K deficiency were different among parts of cotton seedlings. The levels of ZR- and iPA-type CKs decreased in leaves and xylem sap under K deficiency, but increased in the root tissue (albeit insignificantly for the iPA type in Y and inverted Y grafts). Salama and Wareing (1979) studied the effects of low supply of nitrogen (N), phosphorus (P), and K on endogenous CKs in sunflower (*Helianthus annuus*), and found that not only low N and low P but also low K decreased the levels of CKs in the roots. The difference between the present results and those of Salama and Wareing (1979) may be due to interspecific differences (cotton versus sunflower) in responses to K deficiency and the different intensities of K deficiency applied (~1/100 versus 1/10).

Recent studies showed potential co-regulation of the ABA and CK status in plants. Changes in ABA status can regulate shoot CK concentrations via altering cytokinin oxidase activity (Vysotskaya et al., 2009). Root overexpression of the *ipt* gene can decrease ABA accumulation in roots, xylem sap, and the mature leaf of salinized plants (Ghanem et al., 2011a). In the present study, significant negative relationships of ABA with ZR- and iPA-type CKs were also found in the organs determined (data not shown). However, no causal relationship, or direct interaction, between them can be discerned.

**ABA and CKs are responsible for premature senescence induced by K deficiency**

Ghanem et al. (2008) and Albacete et al. (2010) have demonstrated that CKs in tomato was the hormonal parameter best related to photosystem II efficiency (*Fv*/*Fm* indicative of leaf senescence) under salinity. In the present study, a close negative correlation of photosynthesis rate (*Pn*) in the fourth main-stem

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**Fig. 4.** Effect of K deficiency on cytokinin (including the ZR-and iPA-type) levels of cotton Y graft (scion+scion/rootstock) at the 8–9 leaf stage. Grafting was performed hypocotyl-to-hypocotyl at the 1-leaf stage of the rootstock and the cotyledonary stage of the scion. Grafts were maintained in nutrient solution with 0.1 mM K during establishment, and transferred to solutions with either 2.5 mM (a) or 0.03 mM K (b) after establishment. The ZR- and iPA-type concentrations (ng g⁻¹ FW) in roots (R) and the youngest fully expanded leaf (L), and xylem ZR- and iPA-type delivery rates (ng plant⁻¹ 24 h⁻¹) below (B) and above the graft union (A) were determined. For each K level, means of the same sampling part (i.e. roots, leaf, and xylem sap collected both below and above the graft union) followed by the same letter are not significantly different according to Duncan’s multiple range test, *P* < 0.05, *n*=4.
leaf from the apex (Li et al., 2012), indicative of leaf senescence, with leaf ABA concentrations, scion xylem ABA delivery rates, and ratios of ABA/(ZR+iPA) type in leaves and xylem sap under K deficiency were found; a close positive correlation was also noted between Pn and CK levels in leaves and xylem sap (Figs 7, 8).

In previous work (Li et al., 2012), a close positive relationship between leaf K content and senescence induced by K deficiency was observed. In order to investigate whether leaf K content influences leaf senescence via phytohormones, K content in the fourth main-stem leaf from the apex (Li et al., 2012) was correlated with ABA and CK concentrations in the same leaf under K deficiency. There was a significant negative relationship between K content and ABA concentration and a significant positive relationship between K content and ZR-type concentration (except standard grafting; Fig. 9). In addition, significant relationships were found between K deliveries and phytohormone levels in rootstock xylem sap of standard (except iPA-type CKs) and inverted Y grafts and in scion xylem sap of Y grafts (data not shown). However, there were no significant relationships between root K content and ABA and CK concentrations in root tissues.

**Feedback regulation of ABA and CK levels in leaves and scion xylem sap (collected above the graft union)**

**is guaranteed by the different grafting types**

Grafting is a useful tool to test whether there was a feedback regulation of some physiological responses, including leaf senescence. For the seedlings exposed to K deficiency, the rootstocks have a tendency to alter phytohormone levels in leaves and scion xylem sap in some cases (Table 1), even significantly for the leaf ABA concentration of a CCRI41 scion grafted onto a SCRC22 rootstock (compared with CCRI41 self-graft; Fig. 1b), but the scion cultivars explained a higher percentage of variation within grafting treatments under K deficiency irrespective of grafting types. No interactions were found between rootstock and scion in the standard grafting experiment regardless of plant organs and phytohormone types (Table 1), indicating that rootstock and scion are autonomous to a great extent in terms of ABA and CK levels.

The levels of ZR- and iPA-type CKs were consistently lower in leaves and xylem of CCRI41 scions than those of SCRC22 scions, and the leaf ABA concentrations and scion xylem ABA flux were consistently greater in grafts with CCRI41 as scions than those with SCRC22 as scions. These results revealed that the cotton shoot can modify (i.e. feedback regulate) the import of phytohormones from the root, as reported in Beveridge et al. (1997) and Foo et al. (2007) for pea and Arabidopsis, and the leaf phytohormone concentrations. Furthermore, it was noticed that it is the xylem phytohormone concentration rather than sap volume that was regulated by shoot cultivars. With respect to seedlings grown under K sufficiency, although significant genotypic differences in scion xylem CK flux were observed in some cases, the leaf CK concentrations were similar between CCRI41 and SCRC22 scions (Figs 2a, 4a, 6a; Table 1).

Consistent with the present results, Gan and Amasino (1995) and Faiss et al. (1997) found that the CK-overproducer rootstock failed to delay leaf senescence via a grafting study. Due to the absence of data for xylem CKs in grafts which they performed, it
remained unclear whether or not there was a feedback regulation of CKs exported from roots by the shoots. However, the present results are different from the classic notion that the roots play an important role in regulation of leaf senescence by delivering hormones to shoots (van Staden et al., 1988; McKenzie et al., 1998; Dong et al., 2008; Albacete et al., 2009, 2010; Ghanem et al., 2011), reflecting the diversity and complexity of the long-distance signalling system in higher plants. As for ABA, studies with reciprocal grafts of wild-type plants and ABA-deficient mutants indicated that the mutant rootstocks had no/little effect on ABA concentrations in xylem sap (Dodd et al., 2009) and in the leaves (Holbrook et al., 2002) of wild-type scions, which are similar to those in the present study.

### Fig. 6. Effect of K deficiency on cytokinin (including the ZR-and iPA-type) levels of cotton inverted Y graft (scion+scion/rootstock) cotton at the 8–9 leaf stage. Grafting was performed hypocotyl-to-hypocotyl at the 1-leaf stage of both the rootstock and scion. Grafts were maintained in nutrient solution with 0.1 mM K during establishment, and transferred to solutions with either 2.5 mM (a) or 0.01 mM K (b) after establishment. The ZR- and iPA-type concentrations (ng g⁻¹ FW) in roots (R) and the youngest fully expanded leaf (L), and xylem ZR- and iPA-type delivery rates (ng plant⁻¹ 24 h⁻¹) below (B) and above the graft union (A) were determined. For each K level, means of the same sampling part (i.e. roots, leaf, and xylem sap collected both below and above the graft union) followed by the same letter are not significantly different according to Duncan's multiple range test, P < 0.05, n=4.

The site of feedback regulation of xylem ABA and CK levels is the hypocotyl.

Xylem sap was collected below and above the graft union, and the phytohormone levels were measured separately. This permits the site of feedback regulation to be deduced more precisely. The three types of grafting were all performed hypocotyl-to-hypocotyl. Because scions did not influence the root (rootstock) ABA and CK concentrations (Figs 1b, 2b, 3b, 4b, 5b, 6b), it is postulated that the site of feedback regulation of xylem phytohormones by scion lies beyond the root tissue. For standard grafts, no significant differences in xylem ABA and CK delivery between rootstock (collected below the graft union) and scion...
Phytohormonal involvement in feedback regulation of cotton leaf senescence

It is considered that when/after xylem phytohormones passed through the graft union from rootstock(s) to scion(s) in Y and inverted Y grafts, they experienced great changes in metabolism, and/or exchanges with xylem parenchyma cells via the action of feedback signal(s). This presumption can at least partly explain the imbalance between rootstock(s) and scion(s) within either a Y graft or an inverted Y graft in terms of xylem phytohormone delivery rates (Figs 3–6), and is supported by the literature. Sauter and Hartung (2002) demonstrated that the lateral transport of ABA in the stem (from the stem parenchyma to the xylem, and vice versa) may contribute to modifying the xylem ABA levels, and xylem sap can be enriched with ABA sourced from xylem parenchyma cells by the higher xylem pH (Li et al., 2011). Moreover, it is known that CKs may be modulated along their transport pathway, for example by movement from xylem to parenchyma cells in the stem or petioles (Singh et al., 1992). Future studies need to confirm the site of feedback regulation of xylem phytohormones further by using interstock grafting and top grafting (on the main stem above the cotyledons, as opposed to the hypocotyl in the present study), and the physiological and molecular mechanisms underlying the changes in xylem phytohormones levels within hypocotyl tissues.

Fig. 7. Relationships between leaf ABA, ZR-, and iPAs-type concentrations, and ABA/(ZR+iPA) ratios (x) with photosynthetic rate (Pn) of the youngest fully expanded leaf (the fourth leaf from the top of the plant) grown under K deficiency (0.03 mM for standard and Y grafts, and 0.01 mM for inverted Y grafts). Open and filled circles denote CCR141 and SCRC22 scions of standard grafts, respectively; open and filled triangles denote CCR141 and SCRC22 scions of Y grafts; and open and filled inverted triangles denote CCR141 and SCRC22 scions of inverted Y grafts. Each point represents the mean of a grafting treatment averaged across three or four replicates, and each replicate consisted of 4–20 plants. The linear regressions were fitted with Sigmaplot 11.0.
The mechanism of feedback regulation of leaf ABA and CK concentrations remains unclear

Theoretically, the level of phytohormones in leaf is regulated at diverse steps, including de novo synthesis, activation, conjugation, and degradation, as well as local and long-distance transport. Since the differences in xylem CKs levels between CCR141 and SCRC22 scions were similar to those differences in leaf CKs concentrations under K deficiency, we cannot exclude the xylem contribution to leaf phytohormone status by long-distance transport in the present study.

The capacity of the leaf to biosynthesize ABA and CKs has been well demonstrated (Miyawaki et al., 2004; Endo et al., 2008). Furthermore, both ABA inactivation (the conjugation with glucose by ABA glucosyltransferase to form ABA glucose ester; Xu et al., 2002) and CK degradation by cytokinin oxidase (Vysotskaya et al., 2009) can occur in the leaf. Therefore, further metabolic studies such as on de novo synthesis, activation, conjugation, and degradation, and local and long-distance transport need to be undertaken to find the exact mechanism underlying the feedback regulation of leaf phytohormone concentrations.

Conclusion

K deficiency strongly increased ABA levels in roots and xylem sap of cotton seedlings and decreased those of ZR- and iP-type CKs in xylem sap and leaves. Correlation analysis indicated that ABA, ZR-, and iP-type levels in leaves and scion xylem sap (collected above the graft union) under K deficiency were closely associated with leaf senescence. The results of both standard (one scion grafted onto one rootstock) and Y (two scions grafted onto one rootstock) or inverted Y (one scion grafted onto two rootstocks) grafting showed that the ABA and CK levels in leaves and scion xylem sap were feedback regulated by scion cultivars under K deficiency. The main action site of basipetal feedback signal(s) involved in xylem phytohormones is the...
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Rootstock hypocotyl (below the graft union) in standard grafting and the scion hypocotyl (above the graft union) in Y and inverted Y grafting. The target of this feedback signal(s) is more likely to be the changes in xylem phytohormones within hypocotyl tissues rather than the export of phytohormones from the roots. The mechanism of feedback regulation of leaf ABA and CK concentrations remains unclear. The results of this study have provided an improved understanding of communication between shoot and root, and are beneficial for the development of approaches to managing this problem.

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Fig. 9. Relationships between K content (x) and ABA, ZR-, and iP-type concentrations in the youngest fully expanded leaf (the fourth leaf from the top of plant) grown under K deficiency (0.03 mM for standard and Y grafts, and 0.01 mM for inverted Y grafts). Open and filled circles denote CCR141 and SCRC22 scions of standard grafts, respectively; open and filled triangles denote CCR141 and SCRC22 scions of Y grafts; and open and filled inverted triangles denote CCR141 and SCRC22 scions of inverted Y grafts. Each point represents the mean of a grafting treatment averaged across three or four replicates, and each replicate consisted of 4–20 plants. The linear regressions were fitted with Sigmaplot 11.0.
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