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# Influence of *Methylobacterium* on iron translocation in plants

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**Abstract** Iron metabolism in plants is essential to maintain optimal growth and iron nutrition is dependent on uptake of iron from the environment and movement of iron in the plant tissues. We have examined the translocation of iron in plant leaves following foliar application of FeEDTA to *Vicia faba* and *Zea mays*. Using radiolabeled iron, we observed that iron translocation is stimulated by products of *Methylobacterium mesophylicum* and by the cytokinin, kinetin. When cytokinins were applied to leaves along with $^{55}$FeEDTA, the rate of iron translocation was greater than in controls without cytokinin addition. Since recent studies indicate that *M. mesophylicum* is widely distributed in the environment as a pyllospheric bacterium, this organism may have an important role in enhancing translocation of nutrients in plant leaves.

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## Introduction

Cytokinins are plant hormones that are known to stimulate plant development by promoting cell division, influencing nutrient flow throughout the plant, regulating apical dominance, and retarding senescence (Taiz and Zeiger 1998). The first chemical designated as a cytokinin was kinetin, 6-furfurylaminopurine, which was not of plant origin but a breakdown product from heating DNA (Miller et al. 1955). Another cytokinin is zeatin, *trans* -6-(4-hydroxy-3-methyl-2-butenylamino)purine, and it was initially isolated from immature endosperm of *Zea mays* (Letham 1973). In addition to plants, various rhizospheric microorganisms (Akiyoshi et al. 1987; Srivastava 2002) produce zeatin isomers. The secretion of *trans*-zeatin by *Methylobacterium* (Koenig et al. 2002) is relevant because it is a bacterium commonly associated with plant leaves (Holland and Polacco 1994; Kutschera 2007; Delmotte et al. 2009) and Holland (1997) has proposed that cytokinin production by the endophytic *Methylobacterium* plays a major role in stimulating plant activity. Since iron is an important nutrient for plants and correction of iron deficiency in plants is frequently accomplished by foliar sprays (Hansen et al. 2007), we examined cytokinin-stimulation of iron translocation in leaves of broad bean and corn.

## Iron uptake and translocation in plants

Several studies have examined iron uptake from the environment by plants. Iron in the rhizosphere is acquired by dicotyledonous plants by a processes designated as Strategy I while monocotyledonous plants use Strategy II (Marschner and Römheld 1994). Under iron deficiency stress, Strategy I plants acquire iron by an inducible ferric chelate reductase (Schmidt 2007) and acidify the rhizosphere by an inducible ATPase-driven proton exporter (Wei et al. 1997). Iron-deficient graminaceous plants (Strategy II) secrete phytosiderophores and employ a specific transport system for uptake of ferrated phytosiderophores (Takagi 1976). Ionic iron in the form of $Fe^{+3}$ is not translocated in the plant but citrate and nicotianamine are necessary chelators for iron translocation in xylem sap and leaf apoplasm (Stephan et al. 1996; Bauer and Hell 2007; Kawai and Alam 2007).

While soil applied fertilizers were commonly used to correct iron deficiency in plants, foliar application of iron solutions were used to enhance growth of beans (Rodríguez-Lucena et al. 2010; Neumann and Prinz 1975), and groundnut (Singh and Dayal 1992). Not only did foliar application of iron enhance rice yield (Fang et al. 2008) and juice quality in sugar cane (Pawar et al. 2003) but also it reduced zinc toxicity in tomato plants (Kaya et al. 1999). For economic reasons, ferrous salts are commonly used in foliar applications; however, iron citrate and iron ethylenediaminetetraacetic acid (EDTA) are also effective (Singh and Dayal 1992; Fernández et al. 2004).

## Cytokinin and iron deficiency chlorosis

Several studies addressing correction of iron chlorosis in plants by foliar sprays have focused on some of the mechanisms involved. Pretreatment of chlorotic plants with the auxin transport inhibitor triiodobenzoic acid (TIBA) was reported to enhance translocation of iron in tomato plants and apple seedlings (Kessler and Moscicki 1958); however, TIBA was not an effective pretreatment for iron translocation in lemon trees (Bar-Akiva and Hewitt 1959) or bean plants (Kannan and Mathew 1970). The transport of radioiron from the primary leaf to trifoliate leaves of bean plants was enhanced by pretreatment with kinetin and gibberellic acid as reported by Kannan and Mathew (1970), but these experiments are difficult to interpret because cytokinin may be stored in roots (Noodén and Letham 1993) and transported to the shoot by the xylem (Torrey 1976). As part of its hormonal signaling, cytokinin stimulates the synthesis of chloroplast proteins where the proteins from nuclear genes are synthesized in the cytoplasm and, therefore, cytokinin has been implicated in preventing leaf senescence (Srivastava 2002).

## Evaluation of iron translocation in leaves

For study of iron translocation in broad bean, *Vicia faba*, and corn, *Zea mays*, seeds were germinated and grown in potting soil under greenhouse conditions. Two sets of leaf pairs were removed each with a 3 mm petiole section from a bean plant and assay procedures followed those established by Gersani and Kende (1982). When using corn, secondary leaves were selected when leaves were 15 cm long and procedures of Müller and Leopold (1966) were employed. A solution containing 12 μmoles of kinetin (Sigma–Aldrich, St. Louis, MO) or bacterial culture fluid was applied onto the leaf using a 4 mm plastic loop to retain the fluid before the leaves were incubated in a chamber with 90% relative humidity in the dark at 20°C. After 2 days, 2 μmoles of Fe as FeEDTA containing $^{55}FeCl_3$ (0.34 μCi; Amersham, Piscataway, NJ) was applied as 20 μl to the designated site and incubated in the chamber for an additional 5 days. With the leaf pairs of bean, cytokinin, bacterial culture extract, and radioiron were applied to the same leaf or to opposite leaves while with corn, cytokinin, bacterial extract and radioiron additions were either on the same or opposite side of the midrib. For the generation of metabolic products secreted by the bacteria, *M. mesophylicum* was grown under established procedures (Holland and Polacco 1992) and the spent culture fluid was dried by lyophilization. Metabolic products of *M. mesophylicum* were dissolved in warm ethanol, desalted and 2 μl of the aqueous extract was applied onto bean or corn leaves at a dose equivalent to 10 ml of culture fluid per leaf.

To establish the location of radioiron, leaves of *V. faba* or *Z. mays* were placed on autoradiographic film (Kodak XK-1) for 7 days at −30°C and film was

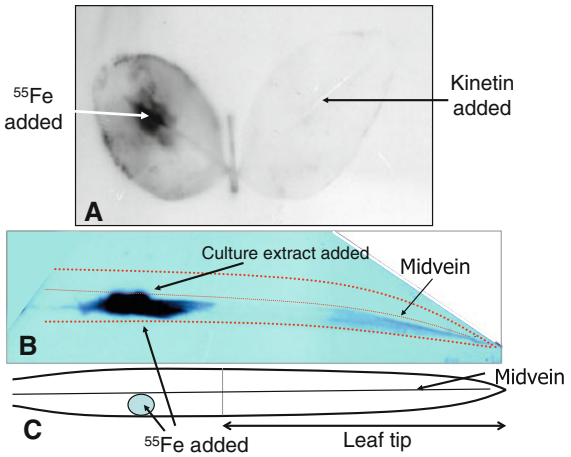


**Fig. 1** Distribution of radioiron in bean and corn leaves. **a** Autoradiogram after $^{55}$FeEDTA and kinetin were added to opposite leaves of the bean leaf pair. The darkened region of the petiole section and leaves indicate the presence of radiolabeled iron. **b** Autoradiogram after $^{55}$ FeEDTA and culture extract from *M. mesophylicum* were added at the site as indicated. **c** A model of the corn leaf

developed by standard methods. Following radioautography, samples were taken from the bean leaves at the site where radioiron was applied using a 8.5 mm diameter cork borer and the petiole section with the entire leaf not receiving radioiron were collected. With corn, the leaf apical to the site receiving radioiron was collected and this region is identified as "Leaf tip" in Fig. 1. Plant tissue was digested with tissue solubilizer TS-2 (Research Products International Corporation, Mount Prospect, IL) and Biofluor (New England Nuclear, Boston, MA) was added to each vial to facilitate the measurement of $^{55}$Fe by liquid scintillation.

## Impact of cytokinin on iron translocation in bean and corn leaves

The movement of $^{55}Fe^{3+}$ applied to bean and corn leaves was influenced by the presence of cytokinins. As observed from the autoradiograph in Fig. 1, radioiron moved from the site of application to the periphery of both bean leaves and to the petiole when kinetin was added to the leaf opposite from the one receiving radioiron. With corn, radioiron moved toward the tip of the leaf when both radioiron and bacterial extract were added at the same side site on the leaf (Fig. 1).

The effectiveness of cytokinin in promoting iron translocation in bean leaves is summarized in Fig. 2. When kinetin or culture extract from *M. mesophylicum* were applied to the same site as radioiron, translocation of radioiron was enhanced with only 6.7 and 2.3%, respectively, of radioiron remaining at the site. In comparison, 28.7% of radioiron remained at the site with the water control. With kinetin, bacterial extract or water added to the leaf opposite of the one receiving radioiron, the amount of radioiron remaining at the site of application was 40.7, 48.7 and 47.6%, respectively. Another way of evaluating translocation of radioiron in response to cytokinins is to examine the amount of radioiron moved into the petiole and opposite leaf, see Fig. 3. With kinetin and culture products from *M. mesophylicum* applied to leaves opposite from the one receiving the radioiron, 40.0 and 33.4% of radioiron was moved into the leaves receiving the cytokinins. With the control where water was added to the leaf opposite the one receiving radioiron, only 1.7% of the radioiron was moved into the opposite leaf. Significantly, small quantities of raidoiron were translocated into the opposite leaf and petiole section if kinetin, bacterial culture extract, or water was added to the same leaf as the radioiron.

With corn leaves, the translocation of radioiron from the site of application to the apical part of the leaf was observed when cytokinin was added to the same site as the radioiron (Fig. 4). Kinetin and culture extract accounted for 16.9 and 12.8% of the radioiron moving toward the leaf tip when cytokinin

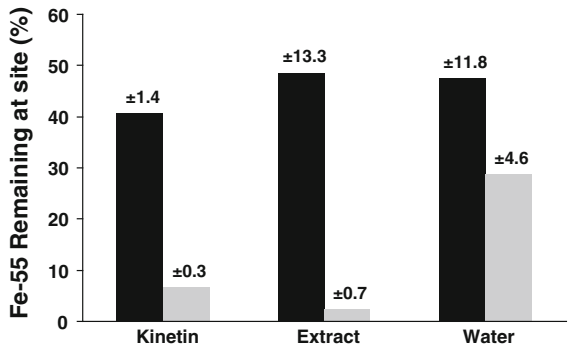


**Fig. 2** The amount of radioiron remaining at the site of application with bean leaves. Additions of kinetin, extract, or water were made to the opposite leaf (*diagonal lines*) or to the same site as radioiron on a leaf (*vertical lines*). Standard deviations of four replica experiments are indicated above each *bar*

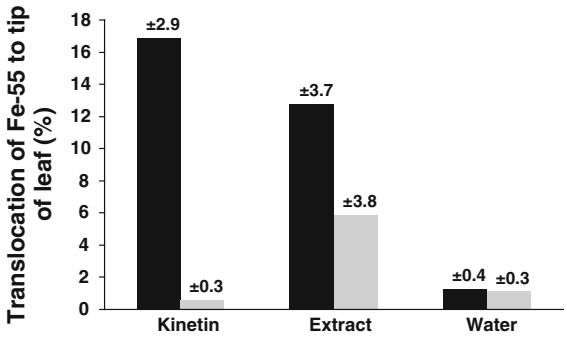


**Fig. 3** The amount of radioiron translocated in bean leaves following cytokinin additions. Additions of kinetin, extract, or water were made to the opposite leaf (*diagonal lines*) or to the same site as radioiron on a leaf (*vertical lines*). Standard deviations of four replica experiments are indicated above each *bar*

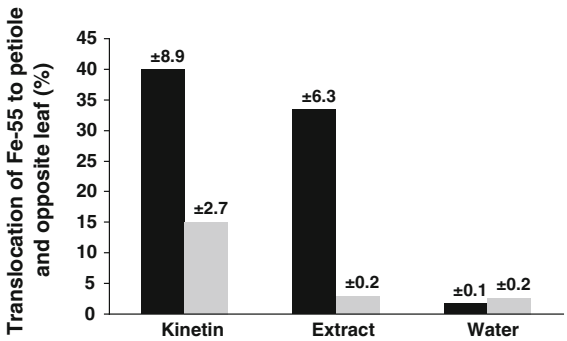


**Fig. 4** The amount of radioiron translocated to the tip of corn leaves following addition of cytokinins. Additions of kinetin, extract, or water were made on the opposite side of the midrib (*vertical lines*) or on the same side and same site of radioiron addition (*diagonal lines*). Standard deviations of four replica experiments are indicated above each *bar*

was added to the site where radioiron was applied. In comparison, only 1.3% of radioiron was translocated toward the leaf tip when water was added to the same site as the radioiron. If additions of cytokinin were made on the opposite side of the midrib where radioiron was added, there was little to no stimulation of radioiron movement toward the leaf tip.

## Mechanism of radioiron translocation in leaves

Kinetin and culture extract from *M. mesophylicum* stimulates iron translocation in broad bean and corn leaves reveals signaling by the cytokinins. If the cytokinins were applied to the same site as the radioiron, translocation of radioiron was throughout that leaf and away from the site of application. However, if the radioiron was applied to the leaf opposite to the one with the cytokinin additions, significant levels of radioiron were translocated into the leaf receiving the cytokinins. Similarly, the movement of radioiron in corn leaves was stimulated by cytokinins. While signaling within the same leaf may be expected with the application of phytohormones, the signaling from one leaf to the other in the leaf pair experiment suggests long range communication initiated by the cytokinins. The nature of this long range signaling may be physiological in the bean leaves or may be attributed to specific gene expression as is observed in wheat where translocation of iron from vegetative tissue to grain is regulated by *NAM* genes (Waters et al. 2009).

The foliar application of iron as Fe-EDTA with cytokinin-stimulated translocation is a multi-step process. In a humid environment, the Fe-EDTA molecule diffuses across the leaf cuticle (Neumann and Prinz 1975) and iron would be translocated in the leaf apoplasm complexed with either the added EDTA or by citrate and nicotianamine present in the plant (Bauer and Hell 2007). As a response to a signal, chelated iron would migrate toward the region of cytokinin presence where iron would support the iron-requiring metabolic processes. However, Fe-EDTA is not readily diffused through leaf cell walls (Kannan 1969) but reduction by ferric chelate reductase associated with leaf cells would produce $Fe^{2+}$ which is readily transported into the cells (de la Guardia and Alcántara 1996). While direct results are yet to be presented for some of these steps, the movement of iron into leaf fluid has been observed within 3 days following foliar application of iron chelates to tobacco leaves has been documented (Fernández et al. 2004).

## Perspective

There are numerous systems for solute transport and translocation in plants and it would be reasonable to consider that each of these systems for nutrient translocation will have their own characteristics (Curie and Briat 2003; Schmidt 2003). This stimulation of $^{55}$Fe-EDTA translocation in bean and corn

leaves following addition of cytokinin that we report is distinct from the cytokinin-stimulated translocation of nitrogenous compounds (Mothes and Engelbrecht 1961) and movement of [$^{14}$C] α-aminoisobutyric acid in *Brassica campestris* leaves (Kuraishi and Ishikawa 1977). An important component in this cytokinin-stimulated iron translocation is the impact of micro-colonies of *M. mesophylicum* releasing cytokinin as it grows as an epiphyte on plant leaves or an endophyte that enters the leaves through the stomata. Additionally, many species of *Methylobacterium* produce siderophores and the contribution of bacterial siderophore production to iron translocation would merit investigation. The dynamics of the *Methylobacterium*-plant interaction and the role of cytokinin-stimulated iron translocation will be important areas for future study.

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