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Organic Acids and Iron Translocation in Maize Genotypes

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ABSTRACT

Translocation of Fe was studied in WF9 (Fe-efficient) and ys_1/ys_1 (Fe-inefficient) maize (*Zea mays* L.) genotypes. Iron-deficient WF9 translocated more Fe to the tops than Fe-deficient ys_1/ys_1. Malate and citrate contents of root saps increased nearly 2-fold and aconitate increased over 4-fold in both genotypes as Fe of nutrient solutions increased from 0.1 to 3 milligrams per liter. Relative acid contents in root saps were as follows: malate > aconitate > citrate. Citric acid concentrations in stem exudates were nearly the same as in root sap. Malic acid concentrations were considerably lower in exudates than in root saps, and only a trace of aconitic acid was detected in the exudates. The concentration of Fe was 7-fold higher in exudate of WF9 than in exudate of ys_1/ys_1 and the concentration of exudate P was about the same for both genotypes.

Electropherograms of WF9 stem exudates showed that ^{59}Fe moved toward the anode as ^{59}Fe-citrate. Exudates of ys_1/ys_1 contained insufficient ^{59}Fe to produce radiographs. When ^{59}Fe was added *in vitro* to ys_1/ys_1 stem exudate, the ^{59}Fe moved as ^{59}Fe-citrate, indicating that sufficient citric acid was present in the exudate to chelate the Fe. Effectiveness of citric, isocitric, *trans*-aconitic, and malic acids in moving ^{59}Fe electrophoretically in acetate, citrate, isocitrate, *trans*-aconitate, and malate buffers was studied. Malic, acetic, and *trans*-aconitic acids were ineffective in moving Fe from the origin. Citric acid moved Fe anodically whenever present on the electropherogram and successfully competed with the other acids for Fe.

Results with ys_1/ys_1 roots indicate an absence of an efficient mechanism for transporting Fe from cortical cells to the xylem. If Fe can reach the xylem stream, the ys_1/ys_1 genotype should be as efficient as WF9 in moving Fe to the leaves.

In 1932 Rogers and Shive (12) proposed that if organic acids keep Fe mobile in external solutions, they might perform a similar role inside the plant. This idea was reasonable, but only within recent years has evidence accumulated to confirm this view. Present knowledge indicates that citric acid is the major compound involved in translocating Fe in plants (1, 4, 14–18).

Evidence for the participation of citrate in Fe solubility and transport, however, does not rule out the participation of other organic acids with similar chemical structures and related metabolic functions. Aconitic acid is closely related to citrate in these matters. *Trans*-aconitic acid is the predominant acid in maize (5, 11) and accumulates in relatively high concentrations (6). The concentration of *trans*-aconitic acid in plant tissues decreases under conditions of most mineral deficiencies (5) and has been reported to be higher in maize roots grown with adequate Fe (20).

The objectives of this study were: (*a*) to investigate Fe binding properties of organic acids, particularly that of *trans*-aconitic acid compared with citric acid, (*b*) to determine organic acid contents of root saps and xylem exudates of WF9 and ys_1/ys_1 maize, and (*c*) to compare root absorption of Fe and, particularly, Fe release to the xylem by these plants.

MATERIALS AND METHODS

Nutrient Solutions and Plant Growth. Seeds of WF9 and ys_1/ys_1 (*Zea mays* L.) were grown in nutrient solutions consisting of (mg/l): 102 Ca, 55 K, 15 Mg, 39 NO_3-N, 10 NH_4-N, 15 Cl, 14 S, 3 P, 0.36 Mn, 0.20 B, 0.11 Zn, 0.03 Cu, and 0.06 Mo. Iron was added as FeHEDTA.[2] Before plants were placed in the nutrient solutions, the pH of the solution was adjusted to 6.5 with 1 N NaOH. Two 7-day-old seedlings without attached seeds were transferred to each 9-liter plastic container of nutrient solution and grown 10 or 11 days. Plants grown for the collection of exudates were given 1 mg Fe/l of solution for 7 days before they were brought into Fe stress by supplying solutions containing 20 μmoles of EDDHA and no Fe. After 1 day in the EDDHA solutions, both Fe and EDDHA were omitted from the nutrient solutions. In 4 to 5 days, the upper leaves of the plants became very chlorotic, but growth was not affected appreciably. The plants were grown in a controlled light room under 8-hr darkness, 16-hr light at 1500 ft-c and 24 ± 2 C. Procedures for germination of seed and growth of seedlings are described elsewhere (2).

Exudate Collection. Iron-stressed plants were rinsed with deionized water and transferred to "absorption" solutions. Except for Fe, salt concentrations were the same as in the previous solutions. Iron added to the absorption solution was 0.25 mg/l with 25 μc of ^{59}Fe. Exudate collection from maize involved excision at the base of the stalk, leaving about a 5 mm thickness at the base attached to the roots. Xylem exudate from the cut surface was delivered via plastic tubing to tubes held in ice. The plants used for exudate collection were transferred to absorption solutions after 7 hr into the 16-hr light period, and exudates were collected for 12 hr in the dark at 24 ± 2 C.

Root Saps. After exudate collection or termination of an ex-

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[2] Abbreviations: HEDTA: hydroxyethylenediaminetriacetate; EDDHA: ethylenediaminedi(*o*-hydroxyphenyl)-acetate.

periment, roots were rinsed with deionized water, blotted, weighed, and frozen in plastic bags. Root saps were obtained by thawing the roots and expressing the root sap with a Carver[3] press. Citric, malic, and aconitic acids were determined in the root saps directly, and 0.5 ml of root sap was dried in planchets, and the ^{59}Fe was counted in a proportional counter.

Methods of Analysis. Phosphorus was determined by the vanadomolybdophosphate method (10) and Fe by the *o*-phenanthroline method (13). Radioiron was determined by a proportional counter. Citric acid was determined by the pentabromacetone method (8), aconitic acid by an acetic anhydride-pyridine method (7), and malic acid by a modified malic dehydrogenase method (9). Procedures for malic acid determination were as follows: 0.6 ml buffer (0.4 M hydrazine, 1 M glycine, 0.007 M EDTA, pH 9.5, with NaOH), 0.1 ml of β-NAD (40 mg/ml), sample, and water were added to make 1.8 ml of total volume in test tubes. Reactions were started by adding 0.2 ml of malic dehydrogenase containing 125 μmole units/ml (Sigma Chemical Co., St. Louis, Mo.) in 2.8 M ammonium sulfate, pH 6, or by adding 0.2 ml of water, shaking and incubating 30 min at 37 C. The reaction mixture was cooled 6 min at 25 C, and the absorbance was read at 334 nm. Reaction mixtures containing all reagents except malic dehydrogenase were used as blanks. Malic acid concentrations were calculated by reference to known standards.

Electrophoresis of Exudates and Standard ^{59}Fe-Organic Acids. General procedures and apparatus used for electrophoresis have been described (14). In the first experiment, WF9 exudate and ys_1/ys_1 exudate (Table II) were subjected to electrophoresis in five buffers: acetate, citrate, isocitrate, *trans*-aconitate, and malate. All buffers contained 20 mM sodium acetate at pH 5.4, but the last four contained one of the other organic acids at a concentration of 1 μM. Because ys_1/ys_1 exudate contained very little ^{59}Fe, additional $Fe(NO_3)_3$ and ^{59}Fe were added to give 12 μM Fe and radioactivity near that in WF9 exudate. Five strips of Whatman No. 3 MM paper (7 × 57 cm) were saturated with the respective buffers and equilibrated 3 hr. The two exudates were then subjected to electrophoresis on each paper. Further details are given in the legend to Figure 1. After treatment the papers were dried and radiographed with Kodak no-screen x-ray film to determine the ^{59}Fe distribution.

In a second experiment, $Fe(NO_3)_3$, organic acids, sodium acetate (pH 5.4), and ^{59}Fe were mixed to give test solutions, each containing 12 μM Fe, 1 μM organic acid, 20 μM sodium acetate, and radioactivity comparable with that in WF9 exudate (Table III). After equilibration for 24 hr, these solutions were treated electrophoretically, as in the previous experiment.

RESULTS

The ys_1/ys_1 plants were more chlorotic than WF9 plants at most Fe levels, and the weight of ys_1/ys_1 roots was less than the weight of WF9 at all Fe levels (Table I). The contents of aconitic, malic, and citric acids were higher in WF9 than in ys_1/ys_1. The contents of acids increased as the Fe concentration in the nutrient solution increased. The amount of aconitate in the root sap increased over 4-fold from the lowest to the highest Fe level, and the amounts of both malic and citric acids increased nearly 2-fold (Table I).

When exudate was collected (Table II), more roots were used for ys_1/ys_1 than WF9 to ensure adequate exudate volumes from ys_1/ys_1. Aconitic, malic, and citric acid concentrations in root sap were 0.98, 1.56, and 0.37 for ys_1/ys_1, and 0.37, 1.51, and 0.22 μmoles/ml for WF9, respectively. The concentration of aconitic acid was almost nil in exudates (Table II) compared to that of root sap, and malic acid in exudates was about one-fifth that in the root sap. Citric acid levels in exudates and root sap were more nearly comparable.

The concentration of ^{59}Fe in the WF9 exudate (Table II) was nearly 200-fold higher than that in ys_1/ys_1; on a total Fe basis there was approximately a 7-fold difference. The P concentration was about the same in the exudate of the two genotypes (Table II).

The total amount of Fe in or on the root was not limiting. It was nearly twice as great in ys_1/ys_1 as in WF9 (Table III). Approximately 10% of the ^{59}Fe in the root was expressed in the root sap (Table III). The ^{59}Fe content of the root was about the same for both genotypes.

The very low concentration of ^{59}Fe in ys_1/ys_1 exudate (Table II) made it necessary to add more ^{59}Fe label to get adequate

Table I. *Leaf Chlorosis, Root Weights, and Organic Acid Contents of Root Sap of ys_1/ys_1 and WF9 Maize Grown at Four Levels of Iron*

Genotype	Fe in Solution	Chlorosis[1] Rating	Root	Organic Acids in Root Saps		
				Aconitic	Malic	Citric
	mg/l		*g/plant dry wt*	*μmoles/ml*		
ys_1/ys_1	0.1	4	0.167	0.43	1.44	0.21
	0.3	1	0.256	0.70	1.88	0.30
	1	0.5	0.254	1.43	2.85	0.44
	3	0	0.327	1.93	2.79	0.38
WF9	0.1	2.5	0.209	0.59	2.00	0.40
	0.3	0.3	0.319	1.76	2.98	0.64
	1	0	0.414	2.43	3.82	0.69
	3	0	0.442	2.74	3.20	0.68

[1] Chlorosis rating of tops: 0: no chlorosis; 5: severe chlorosis.

Table II. *Organic Acids, P, Fe, and ^{59}Fe Concentrations of Xylem Exudates of ys_1/ys_1 and WF9 Iron-stressed Plants*

Plants were grown under normal conditions (1 mg Fe/l) for 7 days before inducing Fe stress by transferring them to new nutrients lacking Fe for 4 days; ys_1/ys_1 and WF9 top leaves were both chlorotic and contained 56 and 61 μg Fe/g, respectively.

Genotype	Exudate	Root	Organic Acids in Xylem Exudates					
			Aconitic	Malic	Citric	P	Fe	^{59}Fe
	ml	*g/fresh wt*	*μmoles/ml*			*μg/ml*		*cpm/ml*
ys_1/ys_1	15.5	45.9	0.065	0.21	0.43	13.6	0.5	490
WF9	14.6	27.2	0.001	0.51	0.12	11.6	3.2	90,600

Table III. *Fe and ^{59}Fe Concentration in Root Residue and Root Sap of ys_1/ys_1 and WF9 Iron-stressed Plants*

The residue and sap were from plants used to collect stem exudate (Table II).

Genotype	Root Residue		Root Sap
	Fe	^{59}Fe	^{59}Fe
	μg/g	*cpm/g*	*cpm/ml*
ys_1/ys_1	679	39,100	2,400
WF9	352	42,000	4,680

[3] Mention of a company, trademark, or proprietary product does not constitute a guarantee or warranty of the product by the United States Department of Agriculture, and does not imply its approval to the exclusion of other products that may be suitable.

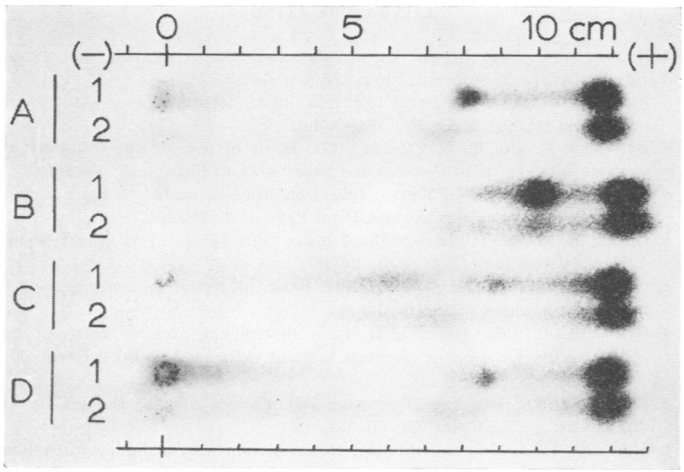

FIG. 1. Radiographs of ^{59}Fe in maize stem exudates after electrophoresis in four buffers. Test solutions: 1: ys_1/ys_1 exudate (Table II) containing additional ^{59}Fe label *in vitro;* 2: WF9 exudate (Table II). Buffers: A: acetate; B: citrate; C: isocitrate; D: *trans*-aconitate. All buffers contained 20 mM sodium acetate at pH 5.4. In addition, buffers B, C, and D contained 1 mM of the indicated organic acid. Conditions: Whatman No. 3 MM paper, 50 μl of exudate per spot, 500 v, 1 hr, room temperature.

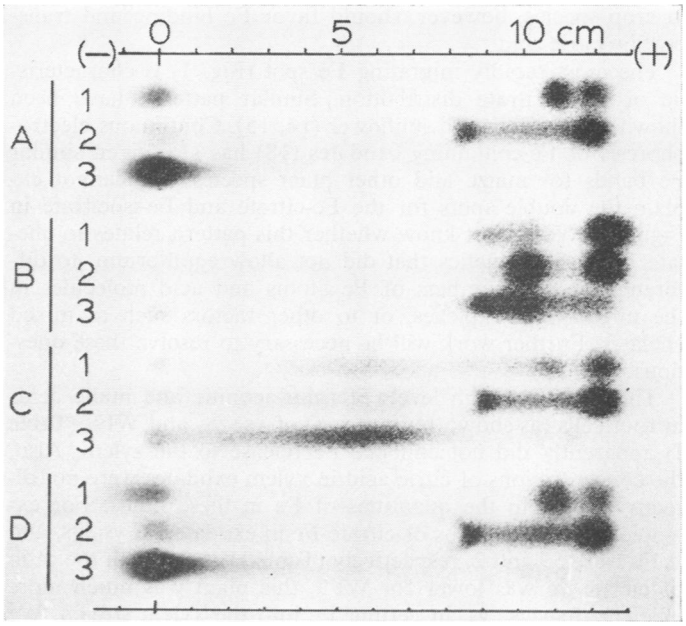

FIG. 2. Radiographs of ^{59}Fe and known organic acids after electrophoresis in four buffers. These solutions contained 12 μM Fe and 1 mM organic acid in 20 mM sodium acetate (pH 5.4): 1: Fe-citrate; 2: Fe-isocitrate; 3: Fe-*trans*-aconitate. Buffers saturating the papers and other conditions are given in the legend to Fig. 1.

film exposures. The aim was to determine whether agents in the exudate of ys_1/ys_1 could bind Fe added *in vitro* and distribute it anodically in a pattern resembling that of WF9. Figure 1 shows the electrophoretic distribution of exudate ^{59}Fe treated in four buffers. The pattern in malate buffer was the same as that for *trans*-aconitate and, therefore, is not shown. In each case, the level of citrate in the exudate was adequate to bind and move Fe anodically to the position of Fe-citrate. The pattern was similar to that shown previously for soybean (17). We cannot explain the appearance of two spots for Fe-citrate and reference is made to this under "Discussion." The inefficient genotype ys_1/ys_1 transported enough carrier in the xylem to bind and keep mobile most of the Fe added *in vitro*.

Acetate and organic acid buffers (A, C, and D, Fig. 1) apparently competed slightly with citrate for Fe as shown by film darkening at the origin and some streaking toward the Fe-citrate spots. The electrophoretic path was obviously cleaner for citrate buffer B.

Figure 2 shows electrophoretic patterns for known Fe-labeled acids. Patterns for acetate and *trans*-aconitate buffers were nearly identical. Acetic and *trans*-aconitic acids were not very effective in moving Fe from the origin (paths A-3 and D-3), although D-3 shows some streaking to about 8 cm anodically. The electrophoresis of Fe-citrate in citrate buffer is on path B-1. Some Fe was located at about 10 cm, but most of the Fe concentration was near 12.5 cm. Isocitrate (path B-2) apparently competed with the citrate buffer, holding some Fe away from citrate and thus accounting for the greater proportion of Fe at 10 cm compared with path B-1. *Trans*-aconitic acid did not compete significantly with citric acid for Fe. Fe-aconitate was spotted at the origin on path B-3. The Fe apparently was released to citrate buffer fairly rapidly, for in the 1-hr electrophoretic period, the Fe moved to positions similar to that on path B-1.

The release of Fe from Fe-aconitate to isocitrate buffer on path C-3 was not as rapid as it was to citrate buffer on B-3. This is apparent from the streaking of Fe in the central area from 3 to 8 cm. Apparently, the equilibrium was not rapid enough on path C-3 to produce the patterns of the pre-equilibrated Fe-isocitrate shown on path C-2.

DISCUSSION

Stem exudates from topped plants have been used extensively to study xylem transport of Fe and other nutrients in plants. Metal transport patterns in exudates are usually considered representative of those in the intact plant. Initial studies suggested that Fe was bound as Fe-malate in soybean exudate (19); however, this was not confirmed in later work. Relatively large amounts of citric acid were found in soybean exudate (4), and Tiffin (14) demonstrated that malate could not compete effectively for Fe held as Fe-citrate. The experimental tests (14) were similar to those used here, except that malate buffer was 10 mM and without acetate. In 10 mM malate, some ^{59}Fe reached the Fe-malate position, but malic acid could not extract Fe from Fe-citrate.

More recent studies (3, 15–18) have confirmed that several crop plants, including maize, transport enough citrate in the xylem to maintain all the Fe chelated as ferric citrate. This study confirms previous findings that Fe-citrate is transported in maize xylem exudate (18) and indicates, in addition, that *trans*-aconitic acid plays only a very minor role, if any, in the solubility and translocation of Fe in maize.

We must emphasize that *trans*-aconitic acid was very low (1 μM) in the xylem exudate of WF9 (Table II). The molar ratio of the *trans*-aconitate to Fe was 1:58. This necessarily precludes significant Fe binding by this acid in the xylem stream.

The equilibrium of isocitric acid with xylem Fe cannot be determined from the present data. Solutions were not analyzed for isocitric acid and, unfortunately, thermodynamic constants for Fe-isocitrate stability are not available. The empirical results from competitive systems shown in Figure 2 indicate, however, that citric acid equilibrates with Fe (from Fe-*trans*-aconitate) more rapidly and transports it with less streaking than does isocitric acid.

The stability values for Fe-isocitrate, when these become known, probably will be near those of Fe-citrate. The greater concentration of citric than of isocitric acid usually observed

in crop species, however, should favor Fe binding and transport by citric acid.

The most rapidly migrating Fe spot (Fig. 1) is characteristic of ferric-citrate distribution. Similar patterns have been shown for soybean and sunflower (14, 15). Continuous electrophoresis of Fe-containing exudates (18) has also given similar Fe bands for maize and other plant species. We cannot explain the double spots for the Fe-citrate and Fe-isocitrate in Figure 2. We do not know whether this pattern relates to chelate formation kinetics that did not allow equilibrium, to differences in the numbers of Fe atoms and acid molecules in the migrating complexes, or to other factors such as mixed chelates. Further work will be necessary to resolve these questions.

The relatively high levels of *trans*-aconitic and malic acids in root cells (as shown by root sap) of ys_1/ys_1 and WF9 (Table I) apparently did not enhance Fe release to the zylem. Also, the concentrations of citric acid in xylem exudates were not directly related to the quantities of Fe in these fluids. For example, the molar ratios of citrate-Fe in exudates of ys_1/ys_1 and WF9 were 59 and 2, respectively (Table II). Although the ratio of citrate-Fe was lower for WF9, this plant was much more efficient than ys_1/ys_1 in getting Fe into the xylem stream. We must conclude that it is not the ratio of organic acid-Fe alone, either in cortical or xylem cells, that accounts for efficient Fe release into the xylem. Other phases of Fe chemistry undoubtedly are involved in the passage of Fe from the cortical cells to the xylem tissues. Brown and Chaney (3) have shown that the amount of citrate transported in xylem exudate was increased by increasing the supply of Fe to soybean and tomato.

The nature of the barrier to Fe translocation in ys_1/ys_1 is unknown. Despite the large amount of Fe on or in the root, only a small amount reached the xylem. The electrophoretic tests revealed that ys_1/ys_1 exudate contained sufficient anionic carrier to bind the Fe that was added *in vitro*. This suggests that if ys_1/ys_1 roots could release Fe to the xylem stream, this genotype would be as efficient as WF9 in moving Fe to the leaves. A better understanding of the chemical phases of Fe movement from the outer root cells to the xylem will be necessary before we know why one genotype is more Fe-efficient than the other.

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