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Full Length Research Paper

The effect of calcium on auxin depletion-induced tomato (*Lycopersicon esculentum* Mill.) pedicel abscission

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Indole-3-acetic acid (IAA) and calcium are the most important factors that instigate plant organ abscission. This study aimed to elucidate the mechanisms that underlie the effects of IAA and calcium on delayed abscission in tomato. The results showed a clear trend towards reduced abscission rates with increased concentrations of IAA, and the applications on pedicel proximal or distal side also resulted in a different abscission. IAA combined with calcium significantly improved inhibition in contrast to IAA only, while IAA combined with magnesium exhibited little increased inhibition. 1-N-Naphthylphthalamic acid (NPA), a polar auxin transport inhibitor, accelerated the abscission. IAA transported basipetally through an assay with 4 mm long pedicel sections indicated that the average transport intensity of [^{3}H]-IAA applied to the distal pedicel end was 65 Bq h^{-1} and the average velocity was 5.29 mm h^{-1}. When the proximal side was incubated in [^{3}H]-IAA, its average transport intensity reduced to 19.53 Bq h^{-1} and the average velocity was only 1.92 mm h^{-1}. Calcium treatment enhanced IAA transportation, as shown by significantly enhancing the transport intensity, but it had no effect on velocity.

Key words: Indole-3-acetic acid (IAA), calcium, abscission, tomato.

INTRODUCTION

Indole-3-acetic acid (IAA) is the most important auxin produced by plants. It plays crucial roles in regulating plant growth and development, including embryo and root patterning, leaf formation, gravitropism, apical dominance, fruit development and the prevention of abscission layer formation and premature fruit drop (Tanaka et al., 2006). Elena et al. (2005) demonstrated that local concentration gradients of auxin (indole-3-acetic acid [IAA]) directed the positioning of primordial organs and stem cell niches, and governed cell division, expansion and separation. In some abscission events, the level of endogenous auxin must fall below a certain threshold in the abscission zone (AZ) to promote abscission (Bangerth, 2000). Guinn and Brummett (1987) reported that a high AZ IAA concentration resulted in fewer cotton balls abscised. However, the AZ IAA concentration in citrus fruit showed an increase before fruit drop (Else et al., 2004). Increases in IAA concentration prior to separation may therefore be necessary for the completion of abscission (Yuan et al., 2002; Else et al., 2004; Abebie et al., 2008).

Meir et al. (2010) divided tomato flower abscission into two phases: early events that probably lead to the acquisition of ethylene sensitivity and abscission competence; and late events when processes occur and lead to the execution of pedicel abscission and the development of the defense layer. The late events, which are ethylene induced, are inhibited by 1-methycyclopropene (1-MCP) pretreatment, while this is not necessarily the case for early events. However, the

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Abbreviations: AZ, Abscission zone; **IAA,** indole-3-acetic acid; **1-MCP,** 1-methycyclopropene; **EDTA,** ethylenediaminetetraacetic acid; **TIBA,** 2,3,5 triiodobenzoic acid; **VP,** verapamil; **TFP,** trifluoperazine.

application of IAA immediately after flower removal inhibits the cascade of abscission events (Addicott, 1982; Brown et al., 2001).

The general rule exists that if IAA is continually available to the AZ region, cells of the abscission layer remain connected, cell separation is inhibited and abscission does not occur (Addicott, 1982; Brown et al., 2001). Leaf abscission can be promoted by removal of the distal region, and delayed by applying auxin at the cut end (Osborne, 1989). Else et al. (2004) reported that polar auxin transport was reduced in fruit pedicels destined to abscind. These results indicated that abscission might be promoted by a decline in normal polar auxin transport.

Auxin (indole-3-acetic acid, IAA) transport serves important role in the allocation of calcium to developing tissues (Banguelos et al., 1987). Dela Fuente and Leopold (1973) first suggested a dependence between polar auxin transport and calcium levels. Their observations indicated that calcium specifically restored the inhibitory effects of ethylenediaminetetraacetic acid (EDTA) on polar transport in sunflower hypocotyl sections. Bangerth (1976) applied the auxin transport inhibitor, 2,3,5 triiodobenzoic acid (TIBA), to tomatoes and apples, which resulted in calcium deficiency-related disorders. The reduced polar auxin transport detected in calcium-deficient sunflower shoots was not of metabolic origin and could be restored by calcium but not by other divalent cations.

It has been demonstrated that calcium plays an important role in auxin transport and secretion (Dela, 1984). The inhibition of abscission can be initiated by incubating explants in a calcium medium or by the direct application of calcium to leaves. Similar effects have been obtained by applying low concentrations of auxin (Xu et al., 2009). Wang et al. (2005) hypothesized that auxin transport stability in tomato pedicels is the key to determining the abscission process, rather than the free IAA concentration in the AZ. However, the mechanism of polar IAA transport by proximal to distal auxin throughout the whole pedicel during abscission remains elusive. Calcium treatment alone or combined with auxin inhibits abscission effectively and is essential to the action of auxin, but the mechanism by which calcium exerts effects on polar IAA transport remains unknown. In the present study aims at evaluating the mechanisms underlying the effects of auxin transport and calcium on abscission delay in tomato.

MATERIALS AND METHODS

Tomato (*Lycopersicon esculentum* Mill. Cv LiaoyuanDuoli) were planted in September 2008 at the Shenyang Agricultural University, China. Plants were cultivated in a glass greenhouse (25 ± 3°C day, 15 ± 3°C night) under natural radiation in 15 cm of soil.

To study petiole abscission, opened flowers were required and these were characterized as follows: all five petals were reflected back from the anther cone, exhibited a fresh yellow color and a floral opening angle of approximately ninety degrees. The flowers were excised from the inflorescence and trimmed immediately into explants under water to reduce the risk of xylem embolism and dehydration. Pedicel explants were trimmed into 2 cm lengths following flower removal.

Chemical treatments and abscission rate investigation

To compare the effect of different incubation directions and IAA concentrations (5, 50 and 100 $\mu g \cdot g^{-1}$ IAA) on abscission, 50 explants were inserted from the proximal or distal end into a Petri dish containing 0.9% agar medium, and ten of such dishes of explants were used as experimental replicates. Different treatments (10 mM $CaCl_2$; 10 mM $MgCl_2$ and 1 mM TFP; 5 mM NPA; 0.5 $mol.L^{-1}$ VP; 10 $mol.L^{-1}$ $CaCl_2$ + 5 $\mu g.g^{-1}$ IAA; 10 $mol.L^{-1}$ $MgCl_2$ + 5 $\mu g.g^{-1}$ IAA; 1 $mol.L^{-1}$ TFP + 5 $\mu g.g^{-1}$ IAA + 10 $mol.L^{-1}$ $CaCl_2$) were applied to each set of dishes to estimate the function of calcium and the IAA transport inhibitor on abscission. One dish without any treatment served as the control. Fifty explants were inserted from the distal end into a Petri dish and ten of such dishes of explants were used as replicates. Dishes were covered with dark film and subsequently placed in a container with a glass cover (40 × 25 × 20 cm^3). The explants were incubated at 25°C.

The number of abscised pedicel explants was recorded at 8, 16, 24, 32, 40 and 48 h following the methods of Wang et al. (2005). Accumulative abscission rates (AR) stood for the percentage of abscised explants in the whole incubation time before investigation. In experiments to determine the extent of basipetal transport, the 4-mm pedicel sections (AZ, including a 2 mm long segment at the joint-like position, proximal and distal sides remained 1 mm long) were placed in reverse orientation into the agar blocks. Routinely, a 0.1 ml droplet of [^{3}H]-IAA was applied to the apical cut surface of each pedicel section using a Hamilton syringe. Calcium and magnesium was added to the IAA solution to evaluate the effect of these ions on polar transport. Each pedicel section was transferred to a new agar block and the time was noted. At 30 min intervals, the agar blocks at the basal end of the pedicel sections were renewed. At the end of the transport period, the pedicel sections were removed from the agar receiver blocks and sectioned into 1 mm segments using a razor blade. Care was taken to avoid the transfer of radioactivity between segments by rinsing the razor blade with deionized water after every cut.

Radioactivity measurements

Agar receiver blocks were placed in plastic scintillation vials with screw lids containing 2 ml of 100% methyl alcohol (MeOH). [^{3}H]-IAA was extracted at room temperature for 24 h. Optima gold scintillation fluid (15 ml) was added to each vial and vortexed. A scintillation counter (Beckman Instruments, Fullerton, CA, USA) on a label ^{3}H channel was subsequently used to count [^{3}H]-IAA levels. Disintegrations per minute were converted to Bq for presentation. Each stem segment was placed in plastic scintillation vials with screw lids containing 2 ml of 100% MeOH and [^{3}H]-IAA was extracted at room temperature for 24 h. [^{3}H]-IAA content in tissue segments were determined as above.

IAA transport velocities and intensities

The intensity (amount of IAA transported per unit of time) and velocity (rate of IAA transport) of polar auxin transport (PAT) in tomato pedicel sections were determined according to the method of Van der Weij (1932). The cumulative radioactivity in the agar blocks over the transport period for each pedicel section was calculated and plotted against time. Times were adjusted to account for the time taken to apply the microdroplets to the pedicel sections.

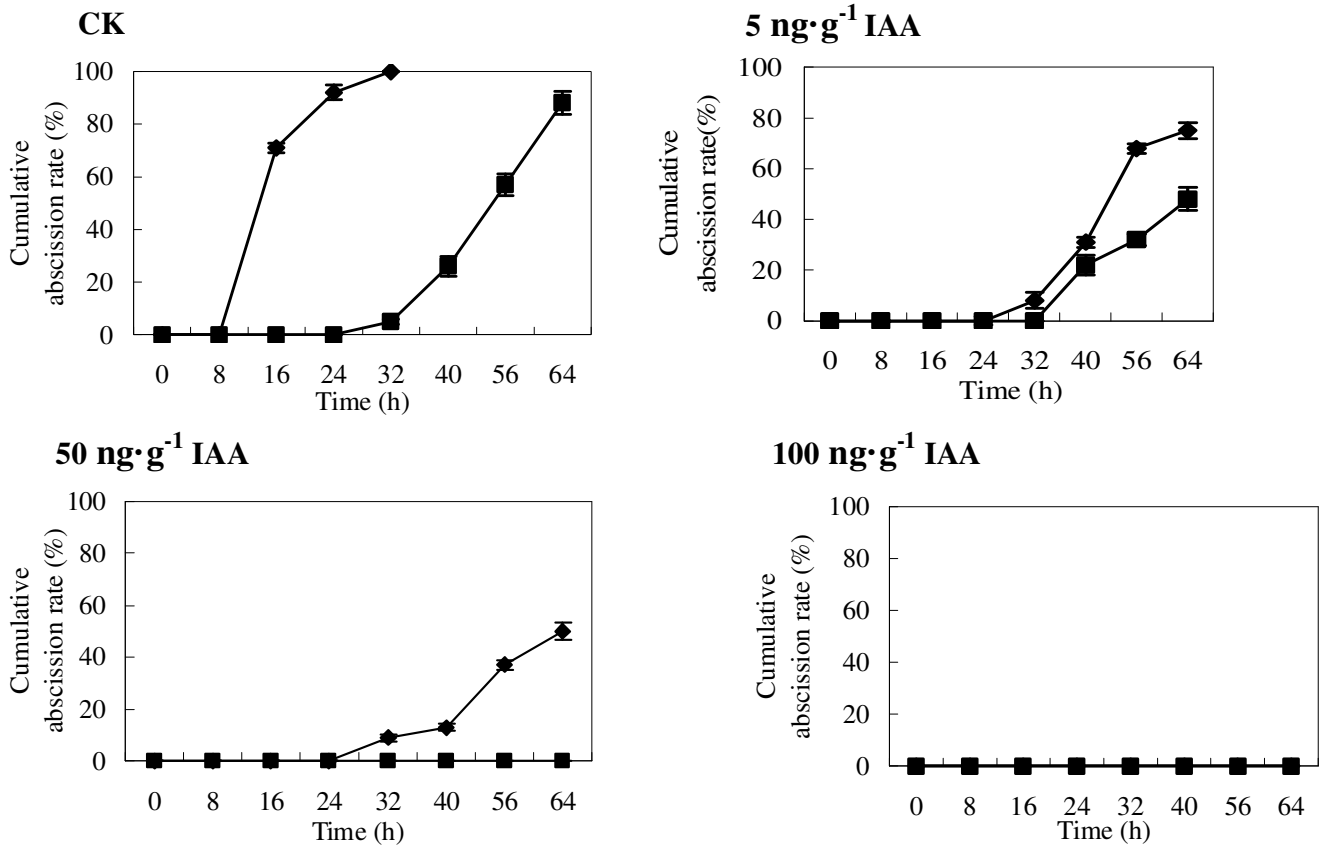

Figure 1. The effects of different IAA concentrations and culture conditions on tomato pedicel explant abscissions sampled at the anthesis. Results show the effects of 0, 5, 50, 100 ng·g^{-1} IAA on abscission rates, respectively. (♦) Proximal pedicel incubated in agar; (■) distal pedicel incubated in agar. Vertical bars indicate ± SE (n = 10).

Linear regression lines were fitted to the transport curves using the least squares method. The slope of the regression line represents the transport intensity*I*(Bq h^{-1}). Individual*I*values were determined for each tissue section and averaged for each treatment. The intercept with the time axis was calculated from the regression equation, which indicates the time at which [^{3}H]-IAA first entered the agar receiver blocks. Transport velocities (mm h^{-1}) were then determined for each tissue section by dividing T_0 by the length of the tissue sections. Velocities were averaged for each treatment.

Statistical analysis

The obtained data were analyzed with Tukey test of comparison of means (P = 0.05) by using the statistical software statistical analysis system (SAS, version 9.1). The transport velocities and intensities experimental unit was ten plants, and each treatment was replicated four times.

RESULTS

The effects of different IAA concentrations and treatments on tomato pedicel abscission

The effects of different IAA concentrations and culture conditions on the abscission of tomato flower pedicel explants are depicted in Figure 1. A clear trend towards reduced abscission was evident when the IAA agar concentration was increased from 0 to 100 ng·g^{-1}. At 32 h, when the control proximal pedicel abscission reached 100%, the decrease in abscission was 84 and 86% after 5 and 50 ng·g^{-1} IAA treatments, respectively. Distal proximal explants were completely inhibited after 50 ng·g^{-1} IAA and proximal and distal pedicel explants were fully inhibited at an IAA concentration of 100 ng·g^{-1}. Furthermore, the inverse incubation of pedicel explants showed effective inhibition of abscission as compared to the controls (proximal part incubated in agar). NPA pretreatment accelerated the time to 50% abscission by nearly 5 h relative to the controls (Table 1).

Calcium and calmodulin enhanced IAA inhibition on explant abscission rates

The effects of calcium nutrition on IAA delayed the abscission of tomato flower pedicel explants as depicted in Table 1. Verapamil (VP), (a calcium channel blocker) reduced the availability of extracellular calcium and trifluoperazine (TFP) (a calmodulin-sensitive calcium-

Table 1. Effects of different treatments on tomato pedicel explant abscission. Numbers followed by the same letter are not significantly different ($P < 0.05$) according to Duncan's multiple range tests.

Treatment	Time to 50% abscission rate (h)
Control	13.8 ± 2.31^{b}
NPA	8.92 ± 1.12^{a}
1 mol.L^{-1} TFP	9.67 ± 1.12^{a}
10 mol.L^{-1} $CaCl_2$	20.18 ± 1.53^{c}
10 mol.L^{-1} $MgCl_2$	14.26 ± 2.92^{b}
5 µg.g^{-1} IAA	43.61 ± 2.89^{d}
10 mol.L^{-1} $Cacl_2$ + 5 µg.g^{-1} IAA	50.87 ± 1.08^{d}
10 mol.L^{-1} $Mgcl_2$ + 5 µg.g^{-1} IAA	46.23 ± 3.01^{d}
1 mol TFP + 5 µg.g^{-1}IAA + 10 mol.$L^{-1}$$Cacl_2$	47.56 ± 2.33^{d}

Data show means ± SE (n = 10).

dependent protein kinase inhibitor). Each treatment significantly accelerated abscission by 4.2 and 4.3 h, respectively (Table 1). The time to reach 50% abscission rates were delayed by approximately 8 h by 10 mM $CaCl_2$. The inhibition of induction was markedly improved by calcium treatment combined with IAA, which markedly delayed abscission and improved 50% abscission rates to 18 h as compare to the sole IAA treatment. Magnesium combined with IAA exhibited little effect on delayed abscission (Table 1). Explants pretreated with 1 mM TFP solution for 1 h, which were then transplanted into calcium and IAA treated agar, exhibited a 50% abscission rate that was similar to that of IAA treatment alone.

Effect of calcium and magnesium on IAA transport and distribution

[^{3}H]-IAA applied to the distal (inverse incubation) or proximal pedicel was transported basipetally through 4-mm long pedicel sections. The linear portions of individual transport curves were used to calculate IAA transport intensity and velocity. Average transport intensity of [^{3}H]-IAA applied to the distal pedicel end was 65 Bq h^{-1} and the average velocity was 5.29 mm h^{-1}. When the proximal side was incubated in [^{3}H]-IAA, its average transport intensity was 19.53 Bq h^{-1} and the average velocity was 1.92 mm h^{-1}. Calcium treatment significantly increased the radioactive IAA transport intensity by approximately 20 Bq h^{-1}, while magnesium only enhanced this by 2 Bq h^{-1} ($P>0.05$). Calcium and magnesium had little effect on the transport velocity, which was enhanced by only 0.32 and 0.37 mm h^{-1}, respectively (Figure 2).

After 4 h of incubation in IAA agar, during the next 30 min, 43 Bq of IAA was detected in tissue within 1 mm of the distal side, while at the [^{3}H]-IAA application site, 22.5 and 4.5 Bq IAA was measured at the AZ and proximal sections, respectively. Calcium and magnesium treatment did not alter the distribution of IAA (Figure 3).

DISCUSSION

Ethylene and auxin (IAA) are important regulators of abscission. IAA delays abscission, whilst ethylene is a potent accelerator of the process. Dogma states that, provided the flux of IAA to the abscission zone region is maintained, cell separation is inhibited and abscission does not occur. In principle, polar auxin transport can be influenced by many factors, including transmembrane pH and electrical gradients as driving forces; these include their carrier kinetics, density, distribution, and the availability of endogenous regulators and the properties of their cognate receptors (Morris and Johnson, 1990). In *Arabidopsis* roots, AUX1 and PIN1 contribute to auxin transport from the vasculature to the root tips through protophloem cells, as the two transporters are located in the plasma membrane at the basal and apical sides, respectively (Jürgen et al., 2006). Differences in IAA transporter distribution and activity exist from the proximal to distal ends of tomato pedicel explants. Our results indicated that the application of IAA from the proximal end of the explants was more effective at delaying abscission than the distal application. Ketsa and Rungruchkanont (2007) reported that 2.4-D showed a higher acropetal IAA transport than NAA and was more effective at inhibiting abscission. The different auxin carrier distribution or activity between the distal and proximal ends may be the result of abscission inhibition when the same concentration of IAA was applied.

Calcium is the most effective abscission inhibition ion (Poovaiah and Leopold, 1973). Calcium binds to cell walls and maintains cell membrane stability, and magnesium has similar attributes. The role of calcium on inhibition is not as simple as a cementing function. The effects of calcium on auxin transport are well documented in gravity-stimulated roots (Blancaflor and Masson, 2003; Perrin et al., 2005). In addition, treatments with auxin transport inhibitors prevented this polar calcium movement and the subsequent gravitropic response (Lee et al., 1984; Lee and Evans, 1985). However, research in

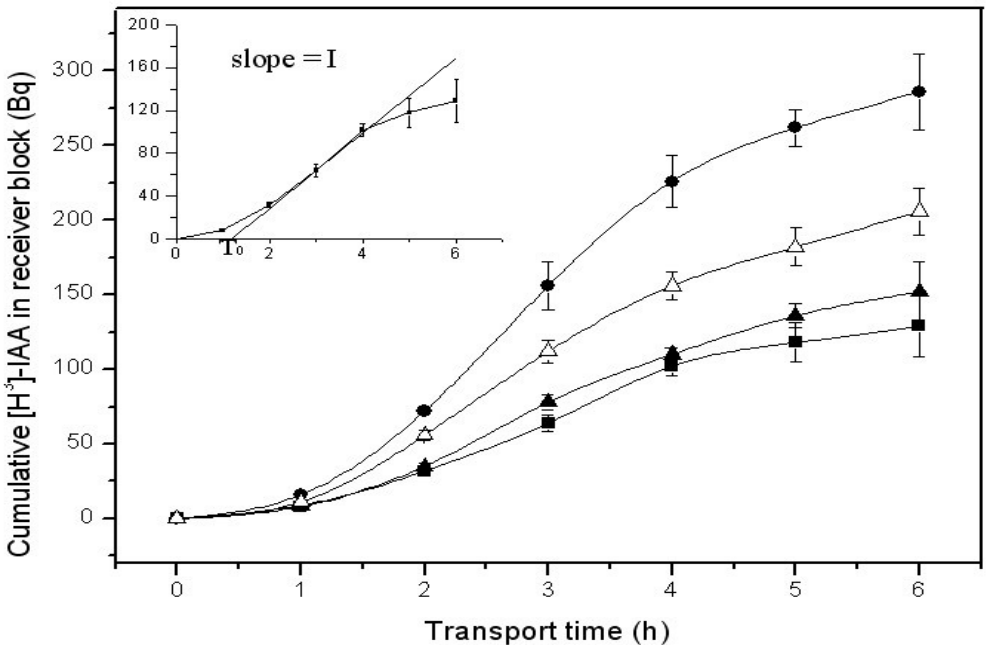

Figure 2. Transport of [^{3}H]-IAA into agar receiver blocks using 4 mm long pedicel sections of tomato variety 'Liaoyuan Duoli'. Linear regression lines were fitted to the transport curves using the least squares method. The slope of the regression line represents the transport intensity I (Bq h^{-1}). Individual I values were determined for each tissue section and averaged for each treatment. T0 indicates that the intercept with the time axis was calculated from the regression equation, which indicates the time at which [^{3}H]-IAA first entered the agar receiver blocks. Transport velocities (mm h^{-1}) were then determined for each tissue section by dividing T0 by the length of the tissue sections. Velocities were averaged for each treatment. (●) Distal pedicel incubated in IAA agar; (■) proximal pedicel incubated in IAA agar; (▲) proximal pedicel incubated in IAA and Mg^{2+} agar; (△) proximal pedicel incubated in IAA and Ca^{2+} agar. Data are the means of ten replicates, with associated standard errors. Vertical bars indicate ± SE (n = 10).

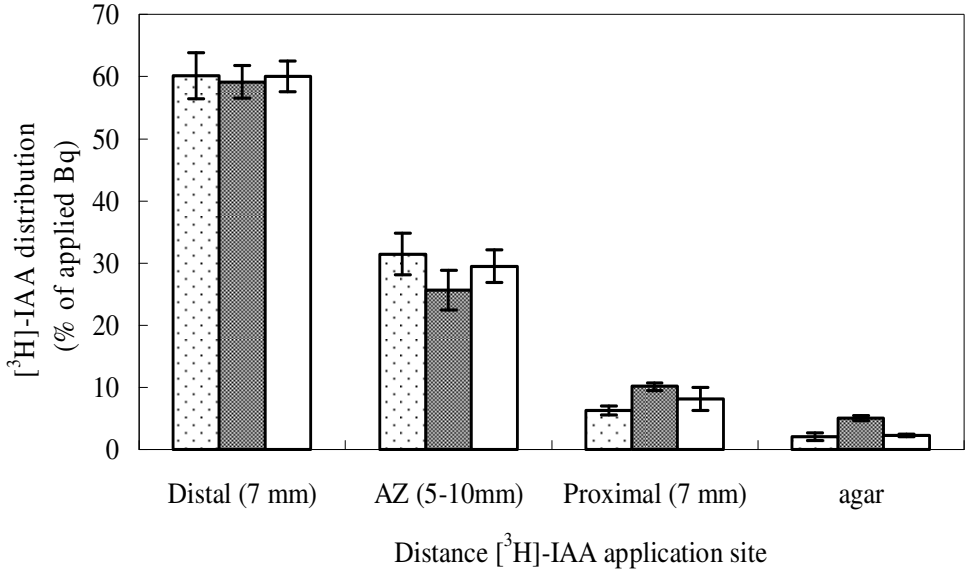

Figure 3. [^{3}H]-IAA distribution at the end of the transport period (>4 h): [^{3}H]-IAA was measured in proximal, AZ and distal sections and received agar. Data are means of ten replicates. (▒) Proximal pedicel incubated in IAA agar; (▓) proximal pedicel incubated in IAA and Mg^{2+} agar; (□) proximal pedicel incubated in IAA and Ca^{2+} agar. Vertical bars indicate ± SE (n = 10).

maize indicated that calcium did not appear to affect the polarity of auxin movement, but clearly increased the total downward polar transport of auxin across the elongation zone of gravity-stimulated roots (Lee et al., 1983) These calcium effects indicate that the auxin transport system depends on the structural or functional features of cellular membranes, which involve calcium in a manner that is analogous to inorganic ion transport (Allan and Rubery, 1991). Hypocotyls of *Cucurbita pepo* L. (zucchini) seedlings showed that calcium reduced the basipetal polar transport of indole-3-acetic and 1-naphthylacetic acids. This evidence demonstrates that during the Ca-depleted or Ca-added state, the intensity of transport is reduced or increased, respectively, but the velocity remains unchanged.

The inhibition of auxin transport by NPA significantly increased ethylene sensitivity and subsequently accelerated abscission. Xu et al. (2009) reported that ethylene signal transduction is required for calcium during abscission. The results were also consistent with an earlier hypothesis that calcium only inhibits stage I of abscission, where IAA exerts its predominant action.

Polar auxin transport was diminished by calcium deficiency and could be stimulated *in vitro* by replacing calcium but not magnesium. VP as a calcium channel blocker changed the extracellular calcium distribution and influenced IAA transport and distribution. It is suggested that the major reason for diminished polar auxin transport in calcium deficient situations is subject to control by calcium under yet unresolved mechanisms. Trifluoperazine (TFP), an inhibitor of the calmodulin-calcium (CaM) complex, accelerated citrus leaf and fruitlet explant abscission, and this acceleration could be counteracted by the simultaneous addition of IAA. In the present study, TFP abolished the effects of calcium on IAA-delayed abscission, which suggest it has a role in IAA-mediated signal transduction.

In conclusion, calcium might adjust auxin transportation and distribution, and the Calmodulin CaM also involved in this process.

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