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ORIGINAL ARTICLE

Subcellular localization of calcium during *Alpinia mutica* Roxb. (Zingiberaceae) style movement

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Abstract The subcellular localization of calcium in *Alpinia mutica* Roxb. during style movement was studied in two morphs. In the styles, Ca-antimonate precipitates (ppts) were principally located in apoplasts, with some minimal accumulation in the nucleus. At different movement, stages of movement, the ppts in the abaxial and adaxial sides changed, and no lateral gradient of ppts in the apoplast was established. The increase or decrease of ppts in the apoplast was not accompanied with equivalent changes in the cytoplasm. These results indicate that calcium could not affect the curvature by inhibiting cell elongation but may play a role in style movement by acting as a secondary messenger. EGTA-treatment affected style movement, providing further evidence supporting a role for calcium as a secondary messenger

Keywords Style curvature · Calcium · Antimonate precipitation · *A.mutica* Roxb

Introduction

Calcium (Ca) is an essential element of growth and development for plants in a myriad of ways, including as an inhibitor of cell elongation (Bennet-Clark 1956; Tagawa and Bonner 1957) and as a secondary messenger in signal transduction systems (Clapham 2007; Shao et al. 2008). Calcium is present in three forms: (1) covalently bound calcium, (2) loosely bound calcium typically associated with fixed and mobile anions, and (3) cytosolic free calcium (Ge et al. 2007). Loosely bound calcium has lower affinity for fixed and mobile anions and functions as an exchangeable form of calcium by transforming into the other forms when and where it is needed (Wick and Hepler 1982). Loosely bound calcium can be examined using a potassium antimonate precipitation method (Wick and Hepler 1982; Zhao et al. 2002).

Previous reports have demonstrated that calcium plays a great role in organ curvature during plant movement (Goswami and Audus 1976; Slocum and Roux 1983; Dauwalder et al. 1985; Gehring et al. 1990; Sinelair and Trewavas 1997; Toyota M et al. 2008). In the cytoplasm, free calcium can act as a secondary messenger involved in phototropism and gravitropism (Poovaiah et al. 1987; Gehring et al. 1990; Sinelair and Trewavas 1997; Plieth et al. 2002; Toyota M et al. 2008). In the cell wall, the pattern of loosely bound calcium distribution changes after gravity stimulation (Slocum and Roux 1983; Dauwalder et al. 1985; Sinelair and Trewavas 1997). It seems that calcium is involved in curvature growth during plant movement resulting from both light and gravity.

The style curvature movements of *Alpinia* plants are very unique in the plant kingdom (Li et al. 2001; Luo et al. 2009). Each style of the two morphs has two curvatures during 1-day flowering, and the directions of the two movements are in opposing directions. The curvature is induced by differential growth of tissues across the style and is characterized by an increase of growth on the convex side of the style. The mechanisms of the growth response are poorly understood, although we have found that auxin

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transport played a significant role during differential growth (unpublished data). In this paper, we investigated the ultramicro-location of Ca during the full flowering process to determine whether Ca^{2+}contributes to style curvature.

Materials and methods

Plant materials

Alpinia mutica Roxb. is a perennial herb, usually 1-3 m tall. The racemes are erect on the terminal of leafy shoots and are over 25 cm in length. The flower has a very unique structure, a conspicuous three-lobed labellum produced by the fusion of two staminodes, which are obovate in shape and yellow tinged with some red coloring. Only one fertile stamen with two anthers develops, and the style extends through the anthers. During blooming, each inflorescence produces one to five open flowers per day, and each flower lasts 1 day. Flowering occurs from May to July. Styles were collected at different moving stages, fixed, sectioned, and observed.

Sample strategy

The styles of each morph in *A. mutica* curved twice. The first one occurred at midnight and finished before daybreak. The second one occurred at about 11:00 and finished at 18:00. To observe the changes of Ca before and after each curvature, we sampled the styles at three movement stages: (1) before the first curvature, at 22:30; (2) after the first curvature, at 06:00; and (3) after the second curvature, at 18:00. Because only the top part (about 25%) of the style had the ability to move (Luo et al. 2009), the sampling were taken from the top of the style that was close to the stigma.

Electron microscopy

The styles were fixed in 2% glutaraldehyde, 2% potassium pyroantimonate, and 75 mM potassium phosphate (pH 7.8) for 4 h at room temperature and washed four to five times in 75 mM potassium phosphate and 2% potassium pyroantimonate (pH 7.8) every 30 min. Samples were subsequently fixed in 1% OsO4 containing 2% potassium pyroantimonate and 75 mM potassium phosphate (pH 7.8) for 12–16 h at 4°C, rinsed in 60 mM potassium phosphate buffer four to five times (30 min changes), dehydrated in a graded ethanol series and embedded in Epon 812 resin. Ultrathin sections were transversely cut using a LKB-V ultramicrotome, stained with 2% uranyl acetate for 15 min, and observed using a JEM-100 CX/II transmission electron microscope.

Controls

Two controls were used: (1) styles not treated with potassium pyroantimonate treatment and (2) sections incubated in 0.1 mM Ethylene glycol-bis(2-aminoethyl-ether)-*N,N,N′,N′*-tetraacetic acid (EGTA; pH 7.8) for 60 min at 60°C to remove the calcium precipitates (ppts).

EGTA treatment on styles

The styles of *A. mutica* were excised from the flowers before the second curvature began, placed in culture dishes, and their bases were sandwiched by two slices of filter papers which were saturated with 1×10^{-4}mol L^{-1} EGTA solution. Here, we borrowed a stigma-anther angle to denote the angle between the stigma and the horizontal line.

Results

The anatomical structure of the style is shown in Fig. 1. Ca precipitates were observed in both the adaxial and abaxial sides. In the styles of *A. mutica*, ppts were located in the style channels, apoplasts and nuclei (Fig. 2).

Ca^{2+} distribution in the styles of the anaflexistylous morph

The two sides of the styles had different growth rates during the style movement. To investigate more closely, we

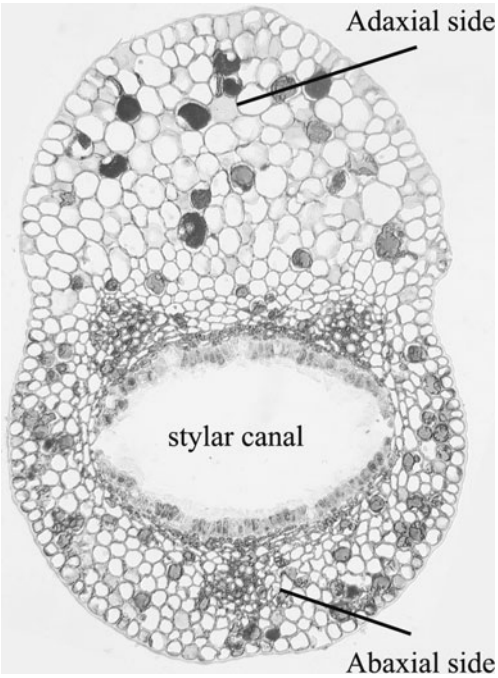

Fig. 1 The anatomical structure of *A. mutica* style

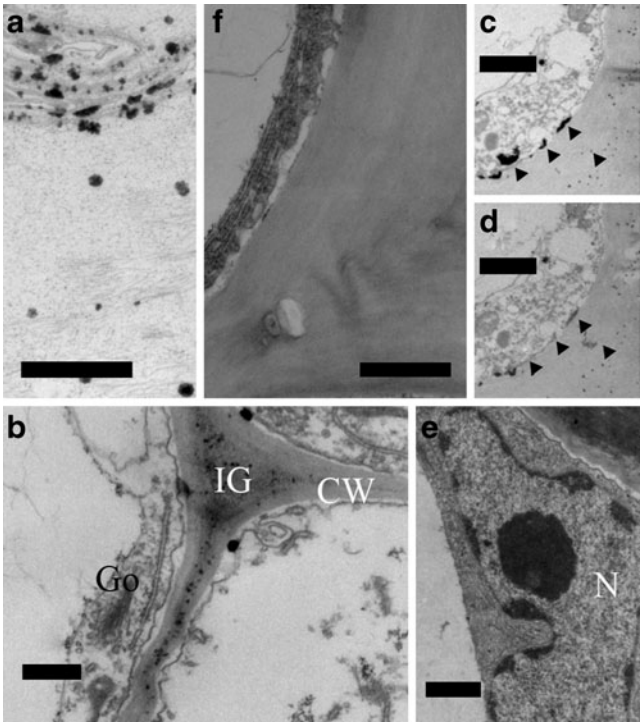

Fig. 2 Calcium distribution in styles (**a**, **b**, and **e**) and EGTA-treated control (**c**, **d**). *CW* cell wall, *IG* intercellular gap, *Go* Golgi apparatus, *N* nucleus. **a** ppts in style channel, **b** ppts in cortic cell, **c** ppts in epidermal cell, **d** ppts in nulcleus. *Bars* **a**, **b** 0.5 μm; **c, d, e** 1 μm

observed the distribution of Ca^{2+} in cells from different side.

The ppts in adaxial cells were shown in Fig. 3. Most of the precipitates were localized to the apoplast, indicating that the majority of Ca resides outside the cell plasma. These precipitates were mainly in the cell wall and intercellular spaces, and a fine line of precipitates is also found along the plasmalemma. Small amounts of Ca were also found in the nucleus, but no Ca precipitates were found in vacuoles. The only major difference in the Ca distribution found in the styles at different stages appears to be a different amount of Ca ppts in the cell wall and intercellular spaces. The distribution of Ca ppts in the abaxial side was shown in Fig. 4. The pattern of ppts in cells was similar to that of adaxial cells: all ppts were in cell walls, intercellular spaces, and nuclei

Figures 3 and 4 show that before the first curvature, the calcium ppts in cell walls and intercellular spaces are both negligible. After the styles complete their first curvature (i.e., before the second curvature), the calcium ppts increased strikingly in cell walls and intercellular spaces. When the second movement was complete, the ppts in cell walls disappeared while levels in intercellular spaces are kept constant.

Ca^{2+} distribution in the styles of the cataflexistylous morph

In the adaxial side, the ppts were abundant in cell walls and intercellular spaces before (Fig. 5a, b) and after the first curvature (Fig. 5c, d). After the second curvature, the ppts in cell walls and intercellular spaces disappeared (Fig. 5e, f). There were about 4 ppts/μm^2 in cell walls and intracellular spaces before the first curvature, and about 5 ppts/μm^2 after the first movement was complete. At the end of the second curvature, the ppts in both organelles disappeared.

The ppts patterns on the abaxial side of the styles were similar to the patterns found on the adaxial side. The cell walls and intercellular spaces had many calcium granules (Fig. 6a) before the first curvature. After the styles finished the first style movement, ppts were also abundant in intercellular spaces and continued to be found abundantly

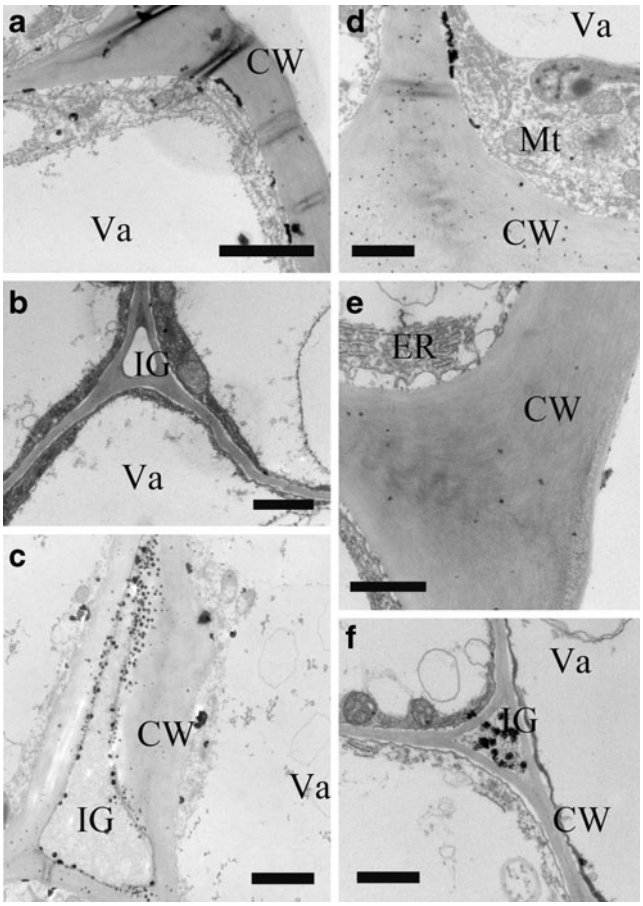

Fig. 3 Calcium precipitates in an adaxial cell of anaflexistylous morph before the first curvature (**a, b**), after the first curvature (before the second curvature; **c, d**) and after the second curvature (**e, f**). *CW* cell wall, *ER* endoplasmic reticulum, *IG* intercellular gap, *Mt* mitochondrion, *Va* vacuole. **a** and **b** No calcium precipitates (ppts) are evident in the cell wall and intercellular gap. **c** and **d** Many calcium granules are distributed in the intercellular gap and cell wall. **e** A few ppts appear in the cell wall. **f** Some ppts appear in the intercellular gap. *Bars* **a** 2 μm; **b-e** 1 μm, **f** 0.5 μm

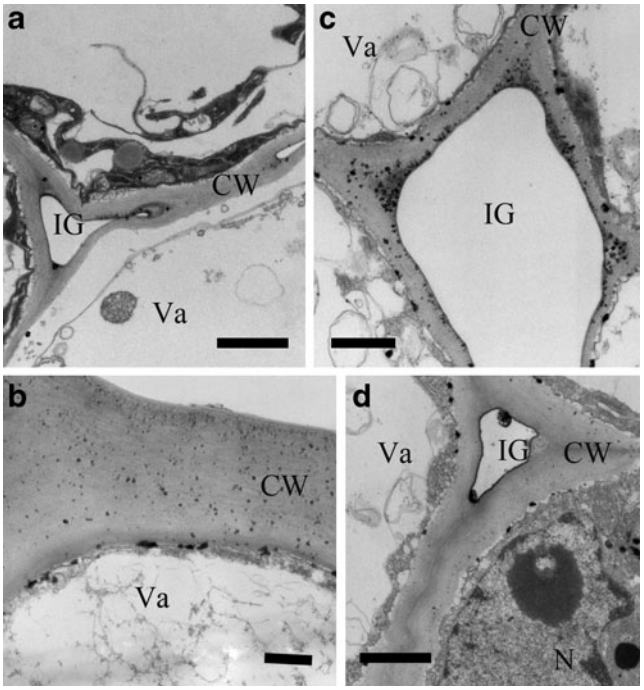

Fig. 4 Calcium precipitates in an abaxial cell of the anaflexistylous morph before the first curvature (**a**), after the first curvature (before the second curvature; **b, c**) and after the second curvature (**d**). *CW* cell wall, *IG* intercellular gap, *Va* vacuole. **a** No calcium precipitates (ppts) are evident in the cell wall and intercellular gap. **b** and **c** Many calcium granules are distributed in the intercellular gap and cell wall. **d** A few ppts appear in the cell wall and intercellular gap. *Bars* **a, b, d** 1.0 μm; **c** 0.5 μm

in cell walls (Fig. 6b, c). However, few ppts were observed when the styles completed the second curvature movement (Fig. 6d).

Effect of EGTA on style movement

After EGTA treatment, style movement was inhibited in both morphs, especially the ana-morph (Fig. 7). The control styles (n=20) of the ana-morph had an average stigma-anther angle of 221.44ºafter the second curvature was complete, while the EGTA-treated styles (n=20) had an angle of only 176.05º (Fig. 7a). The cata-morph styles (n=20) without EGTA treatment had an angle of 139.66º, while the styles (n=20) treated with EGTA had an angle of 168.94º (Fig. 7b).

Discussion

In plant movement, the redistribution of Ca^{2+} within shoots, roots and coleoptiles during curvature growth has been documented (Goswami and Audus 1976; Slocum and Roux 1983; Dauwalder et al. 1985; Gehring et al. 1990; Sinelair and Trewavas 1997; Toyota M et al. 2008). We examined the distribution of Ca^{2+} in styles throughout the whole flowering stages of *A. mutica*, and the results indicate that Ca^{2+} may play a role in style curvature.

Our study shows that loosely bound calcium was mainly localized in the cell walls and the concentration changed in both the abaxial and adaxial sides of styles during bending for both morphs. It seems likely that such Ca accumulations in cell wall and intercellular spaces of styles would interfere with growth in an inhibitory manner because a high Ca concentration in apoplast exerts a general inhibitory effect on elongation growth (Coartney and Morré 1980). This inhibition may be due to the alterations in the mechano-chemical properties of the cell wall via Ca-dependent pectin-protein interactions (Kauss and Glaser 1974), or via antagonistic action of Ca during auxin-mediated growth processes (Cleland and Rayle 1977; Hepler 2005). In

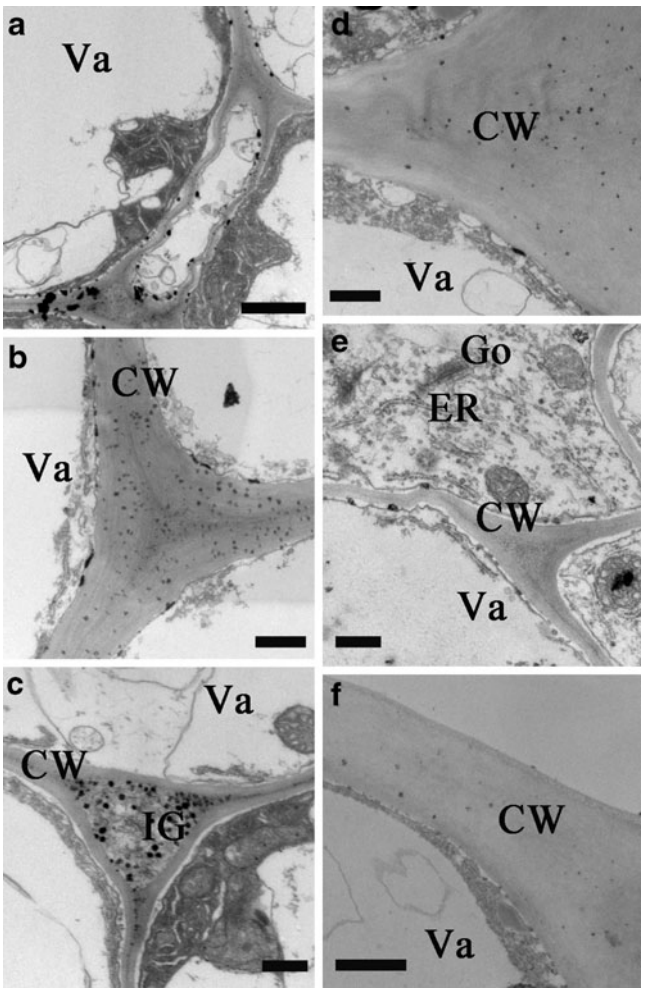

Fig. 5 Calcium precipitates in an adaxial cell of the cataflexistylous morph before the first curvature (**a, b**), after the first curvature (before the second curvature; **c,d**) and after the second curvature (**e, f**). *CW* cell wall, *ER* endoplasmic reticulum, *IG* intercellular gap, *Va* vacuole, *Go*, Golgi apparatus. **a** and **b** Ppts are evident in the cell wall and intercellular gap. **c** and **d** Many calcium granules are distributed in the intercellular gap and cell wall. **e** and **f** A few ppts appear in the cell wall. *Bars* **a, f** 1 μm; **b-e** 0.5 μm

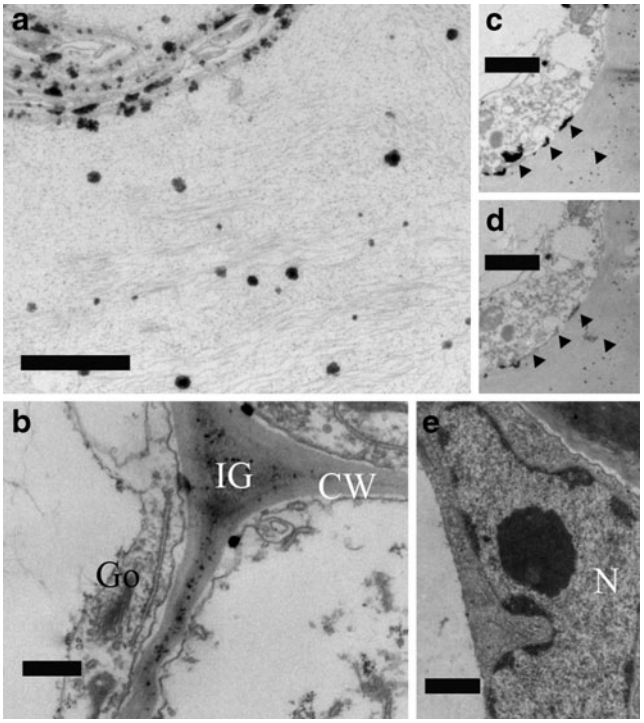

Fig. 6 Calcium precipitates in an abaxial cell of the cataflexistylous morph before the first curvature (**a**), after the first curvature (before the second curvature; **b, c**) and after the second curvature (**d**). *CW* cell wall, *IG* intercellular gap, *Go* Golgi apparatus, *Va* vacuole. **a** Many precipitates are evident in the cell wall and intercellular gap; **b** and **c** Many calcium granules are distributed in the intercellular gap and cell wall. **d** A few ppts appear in the cell wall and intercellular gap. *Bars* **a, b, d** 0.5 μm; **c** 1 μm

addition, there could be interactions between Ca and molecules other than pectins that could contribute to cell wall structure and flexibility. It has been suggested that this inhibitory effect plays a role in gravi-stimulated curvature, because calcium accumulates preferentially in one side of a horizontally positioned organ, and the organ curves toward the side with high Ca in the cell wall (Slocum and Roux 1983). However, analysis of precipitate distribution indicates that no lateral asymmetry of loosely bound calcium is established in cell walls during bending. Symmetrical distribution of Ca between the adaxial and abaxial sides could lead to inhibition of cell growth on both sides of the style, cancelling each other out. Therefore, the role of Ca in style curvature does not appear to involve cell growth inhibition. Similar results were observed in gravi-stimulated corn root (Dauwalder et al. 1985).

In addition to the cell wall, Ca plays a great role as a secondary messenger in cytoplasm. In the phototropic curvature of etiolated oat coleoptiles, Ca^{2+} flux into the side exposed to light before bending was observed. This was regarded as a part of blue light-induced signal transduction (Babourina et al. 2004). Toyota et al. (2008) also found that cytoplasmic calcium increases in response to changes in the gravity in hypocotyls and the petioles of *Arabidopsis* seedlings. During the two style curvature in *A. mutica*, the changes of loosely bound calcium in the cell wall were not accompanied by equivalent changes in the cytoplasm. This suggests that the loosely bound calcium may transform into free calcium leading to changes in the concentration of free calcium in the cytoplasm. This speculation is supported by the change of calcium distribution. For example, a few ppts were found in the apoplast, and no ppts were found in the cytoplasm in the styles of the ana-morph before the first curvature (Figs. 3 and 4). After the first curvature was complete, many calcium granules were distributed in the cell wall and intercellular spaces. We know that there was a lot of free calcium in the cell wall, intercellular space, vacuole, and rough endoplasmic reticulum (Sinelair and Trewavas 1997). The changes of loosely bound calcium in the cell wall and intercellular space in styles could be from the concentration change of free calcium. The free calcium in the cytoplasm and intercellular spaces is believed to be an important secondary messenger. In the two morphs of *A. mutica*, changes in ppts at different movement stages indirectly reflect the change of Ca^{2+} concentration, which may regulate the style curvature as a secondary messenger. The results of EGTA treatment also support this hypothesis, because EGTA, a chelate compound of free calcium, can inhibit style curvature movement (Fig. 7). It should be

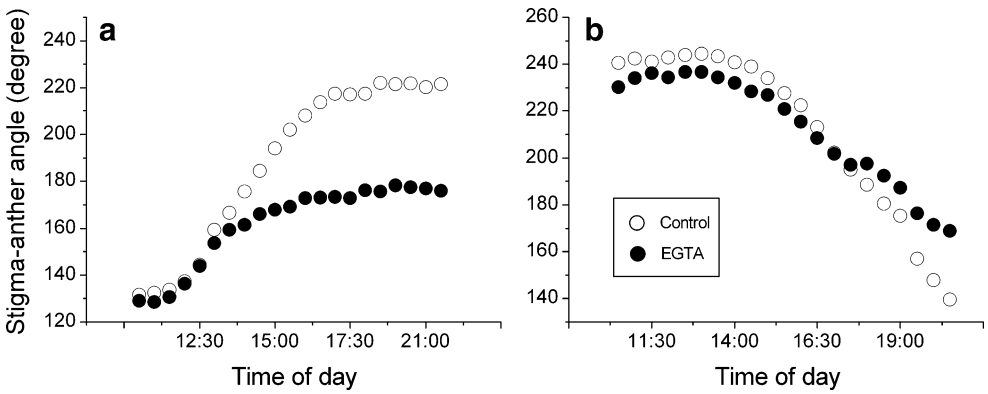

Fig. 7 A time course showing the stigma-anther angle of chemically treated styles of *A. mutica* Roxb. **a** Anaflexistylous morph, **b** Cataflexistylous morph. EGTA (1×10^{-4} mol L^{-1}) treatment can inhibit the style movement of both morphs, especially the anaflexistylous morph

noted that the pattern of changes of loosely bound calcium in the apoplast are different between the two morphs. This suggested that the way Ca^{2+} effects style curvature may be different in the two morphs.

In conclusion, calcium played a role during style curvature movement in *A. mutica* in our study. The role was mediated by changes in ppts distribution at different movement stages. The changes had an important biological significance, which indicates that Ca^{2+} may be a secondary messenger in style movement. The changes do not appear to act by inhibiting cell growth.

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Conflict of interest None

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