Research Article

Exploring the role of auxin in the androgynophore movement in *Passiflora*

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Abstract

The flowers of the species belonging to the genus *Passiflora* show a range of features that are thought to have arisen as adaptations to different pollinators. Some *Passiflora* species belonging to the subgenus *Decaloba* sect. *Xerogona*, show touch-sensitive motile androgynophores. We tested the role of auxin polar transport in the modulation of the androgynophore movement by applying auxin (IAA) or an inhibitor of auxin polar transport (NPA) in the flowers. We recorded the movement of the androgynophore during mechano-stimulation and analyzed the duration, speed, and the angle formed by the androgynophore before and after the movement, and found that both IAA and NPA increase the amplitude of the movement in *P. sanguinolenta*. We hypothesize that auxin might have a role in modulating the fitness of these *Decaloba* species to different pollination syndromes and demonstrate that an interspecific hybrid between insect- and hummingbird-pollinated *Xerogona* species present a heterosis effect on the speed of the androgynophore movement.

Keywords: *Passiflora*, androgynophore, IAA, NPA, thigmotropism.

Introduction

The genus *Passiflora* comprises about 500 species which are mostly woody vines that present a huge diversity in flower shape, colors and sizes. Consequently it is a good model for studying plant-pollinator interactions and co-evolution, since it displays all kinds of pollination syndromes. Although this diversity exists, the flowers always exhibit unique features that characterize and cluster the species in the genus. One of them is the corona filaments, which comprises one or more extra whorls that can play different roles in different species, functioning as nectar guide, or forming a floral tube in flowers adapted for bird pollination, and even serving as a landing platform for insects (Ulmer and MacDougal, 2004). Another common feature is a nectary system containing an operculum and a membrane structure called limen that encloses a nectary chamber. Finally, the androgynophore, a column in the center of the flower that elevates the androecium and the gynoecium, is present in different sizes and even in shapes. In *P. edulis*, which has flowers adapted to insect pollination, the androgynophore is a short and straight column. In contrast, in *P. mucronata*, which is bat pollinated, the androgynophore is a long curved column (Ulmer and MacDougal, 2004).

Recently, we showed that in some *Passiflora* species, the androgynophore can also be a thigmotropic structure, *i.e.*, it has the capability to move in response to touch and the movement is dependent on the direction of the stimulus (Scorza and Dornelas, 2014; Scorza *et al.*, 2014). When mechanically stimulated, the androgynophore inclines to the same side where the stimulus came in about 2 seconds. These species, *P. sanguinolenta*, *P. citrina*, *P. capsularis* and *P. rubra* are in the subgenus *Decaloba*, and the movement is believed to be related to the pollination system of these species. The motile androgynophore would enhance the chances of pollen deposition on pollinators that would approach the flower and touch the column, which in turn would curves in the pollinator's direction upon the mechanical stimulus (Scorza and Dornelas, 2014; Scorza *et al.*, 2014).

Plant fast movements have been widely studied (for reviews, see Braam, 2005; Scorza and Dornelas, 2011), in particular pulvinar movements in Fabaceae species, such as *Mimosa pudica*, where the leaflets of compound leaves shows a fast closing movement in response to touch (thigmonastism) or light regime (photonastism and nyctinastism) (Samejima and Sibaoka, 1980; Moran, 2007; Volkov *et al.*, 2010a). Additionally, carnivore plants such as *Drosera*, *Dionaea* (Droseraceae) and *Utricularia* (Lentibulariaceae), have adapted organs that can produce active movements to capture preys (Sibaoka, 1991; Braam, 2005; Volkov *et al.*, 2008; Singh *et al.*, 2011). Still, stamina of *Portulaca grandiflora* (Portulacaceae), *Berberis*

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canadensis (Berberidaceae), *Opuntia* (Cactaceae) and Loasaceae (Henning and Weigend, 2012) flowers for example, can also bend in response to a visiting pollinator increasing pollen transfer among the flowers, boosting the cross pollination (Jaffe *et al.*, 1977; Fleurat-Lessard and Millet ,1984; Schlindwein and Wittmann, 1997; Scorza and Dornelas, 2011).

The fact that plants are capable of rapidly moving their structures without having any kind of neurological system is attributed to the capability of specific plant cells to swell or shrink by losing water quickly. The maintenance of a differential turgor pressure among plant tissues provides an energy storage that, when released, is capable of moving plant organs within seconds (Sibaoka, 1991; Braam, 2005; Volkov *et al.*, 2008, 2010b).

This turgor pressure is maintained by the activity of proton pumps in the plasma membranes (H^+-ATPase), which are coupled with K^+ and Cl^- fluxes (Samejima and Sibaoka, 1980; Sibaoka, 1991; Fleurat-lessard *et al.*, 1997; Moran, 2007). When the cells get turgid the H^+-ATPase proton pumps is acting extruding H^+ protons outwards of the cell. In order to balance the proton gradient across the membrane, K^+ ions are pumped inwards, increasing the cell's osmotic potential, and therefore, there is a water influx through aquaporins which causes swelling of the cells. When a mechanical or electrical stimulus is applied, an action potential triggers a rapid turgor loss associated with an efflux of ions in the cells that is followed by water (Campbell and Garber, 1980; Moran, 2007; Volkov *et al.*, 2010a).

Auxin is an important coordinator of plant growth and development, and one of the mechanisms where this hormone is involved is the regulation of water and ion permeability to cells (Blatt and Thiel, 1994; Takahashi *et al.*, 2012). In order to clarify how this hormone affects cell turgor regulation, experiments related to the pulvinar movements of *Cassia fasciculata*, *Phaseolus vulgaris* and *M. pudica* have been reported (Watanabe and Sibaoka, 1983; Bourbouloux *et al.*, 1992; Bonmort and Roblin, 1996; Iino *et al.*, 2001; Moyen *et al.*, 2007). Applying indol-3-acetic acid (IAA) to protoplasts of pulvinus of *P. vulgaris* caused swelling of the cells. This was interpreted as enhanced effluxes of K^+ and Cl^- (Iino *et al.*, 2001). When exogenously applied in *M. pudica* and *C. fasciculata*, IAA (indol-acetic acid) increased the angles during the folding of the leaflets (Bourbouloux *et al.*, 1992). 2,4-D, a synthetic auxin, when applied, inhibited the leaflet folding by dark stimulus (Bonmort and Roblin, 1996; Moyen *et al.*, 2007). In all cases the auxin effects seem to be directed towards maintaining a high turgor pressure in the cells (Bourbouloux *et al.*, 1992; Bonmort and Roblin, 1996). These experiments further contributed to the evidence that auxins stimulate the proton extrusion driven by H^+-ATPases in the plasma membrane. Although it was known that auxins have an effect on proton pumps, the mechanisms by which it acts became clear only recently, when Takahashi *et al.* (2012)

showed that auxins mediate H^+-ATPase activation by phosphorylation of the penultimate threonine of the H^+-ATPase during hypocotyl elongation in *Arabidopsis* (Takahashi *et al.*, 2012).

The *P. sanguinolenta* androgynophore is less sensitive to touch than thigmonastic Fabaceae leaflets and also does not respond to a dark or light stimulus (Scorza and Dornelas, 2014). Therefore, we tested the effects of auxin on the movement of the androgynophore of the model species, *Passiflora sanguinolenta.* Among the *Passiflora* species that present a motile androgynophore, we chose *P. sanguinolenta* because this species shows a more conspicuous movement than the others, thus being easier to observe. Additionally, as this species is evolutionary derived in relation to other *Decaloba* species, and adapted to hummingbird pollination (Scorza and Dornelas, 2014), we tested whether the flowers of the interspecific hybrid between *P. sanguinolenta* and the more basal, insect-pollinated *P. capsularis* inherited thigmotropic androgynophore features. We also tested the effects of a specific inhibitor of the auxin efflux, 1-N-naphthylphthalamicacid (NPA) on androgynophore movement. The effect of NPA has not been tested before in the context of active plant movements.

Material and Methods

Plants

Plants of *Passiflora sanguinolenta* Mast., *Passiflora capsularis* L. and their ornamental interspecific hybrid named *Passiflora* 'capsang' (Ulmer and MacDougal 2004) were grown under greenhouse conditions at the Universidade Estadual de Campinas, Instituto de Biologia, Campinas, SP, Brazil.

Auxin and NPA treatments

To test whether the movement is influenced by exogenous application of auxin and an auxin polar transport inhibitor we prepared treatment solutions using indol-3-acetic-acid (IAA, SIGMA), and N-1-napthyl-phtalamic acid (NPA, Chem Service, West Chester, PA). IAA solution was made at a final concentration of 1 mM. The concentration of IAA was chosen based on Moyen *et al.* (2007), where pulvinar movements were inhibited using 2,4-D, a synthetic auxin, at a final concentration of 0.1 mM. They argued that 2,4-D is about 10-fold more effective than IAA, so we used IAA for our experiments at 1 mM. The NPA final concentration was 0.1 mM, based on Petrasèk (2003).

A preliminary test was made spraying NPA at 0.1 mM on the opened flowers still attached to the plant, and the movement tests were made 3 h after spraying. Alternatively, recently opened flowers of *P. sanguinolenta* were removed from the plant and directly transferred to water to avoid desiccation. Subsequently, the tips of the flower pedicels were cut with a sharp knife and immediately

dipped in the treatment solutions (IAA 1 mM; NPA 0.1 mM and water as the control solution). These solutions were in Petri dishes covered with Parafilm, where tiny holes were made to fit the pedicels and keep the flowers in an upright position. The flowers were kept in the treatment solutions for about 3 h, as this was described as a period when, in general, the maximum effect was achieved in pulvinar movement experiments (Moyen *et al.*, 2007).

Recordings and calculations of the duration, angulation and speed of the movement

After treatment with hormonal solutions we transferred the flowers to wet floral foam and removed part of the perianth to visualize the entire androgynophore (Figure 1). The flowers were kept untouched for another 15 min as sometimes we touched the androgynophore when cutting off the perianth, inducing the thigmotropic movement. Finally, we recorded the mechanically induced movement of the androgynophore and calculated the duration, the angle formed between the steady state (before the movement)

and the final state (after the movement) and speed as described previously (Scorza and Dornelas, 2014). The same protocol was used to evaluate untreated flowers of the parental species (*P. capsularis* and *P. sanguinolenta*) and their interspecific hybrid (*P.* 'capsang'). Ten flowers from different individuals were used as replicates for each treatment. The results of the measurements presented in Figures 2-4 are shown as mean values $\pm$ SD and asterisks indicate significant differences at $p < 0.05$ according to Kruskal-Wallis test.

Results

When the flowers of *P. sanguinolenta* were immersed in IAA at a final concentration of 1 mM for 3 h the mean of the angle formed by the androgynophore trajectory before and after the movement was significantly higher than in the control, where flowers were immersed only in water (Figures 1 and 2). The time that the androgynophore took to bend after the mechanical stimulus was also increased in plants treated with IAA (Figure 2). Therefore, we did not
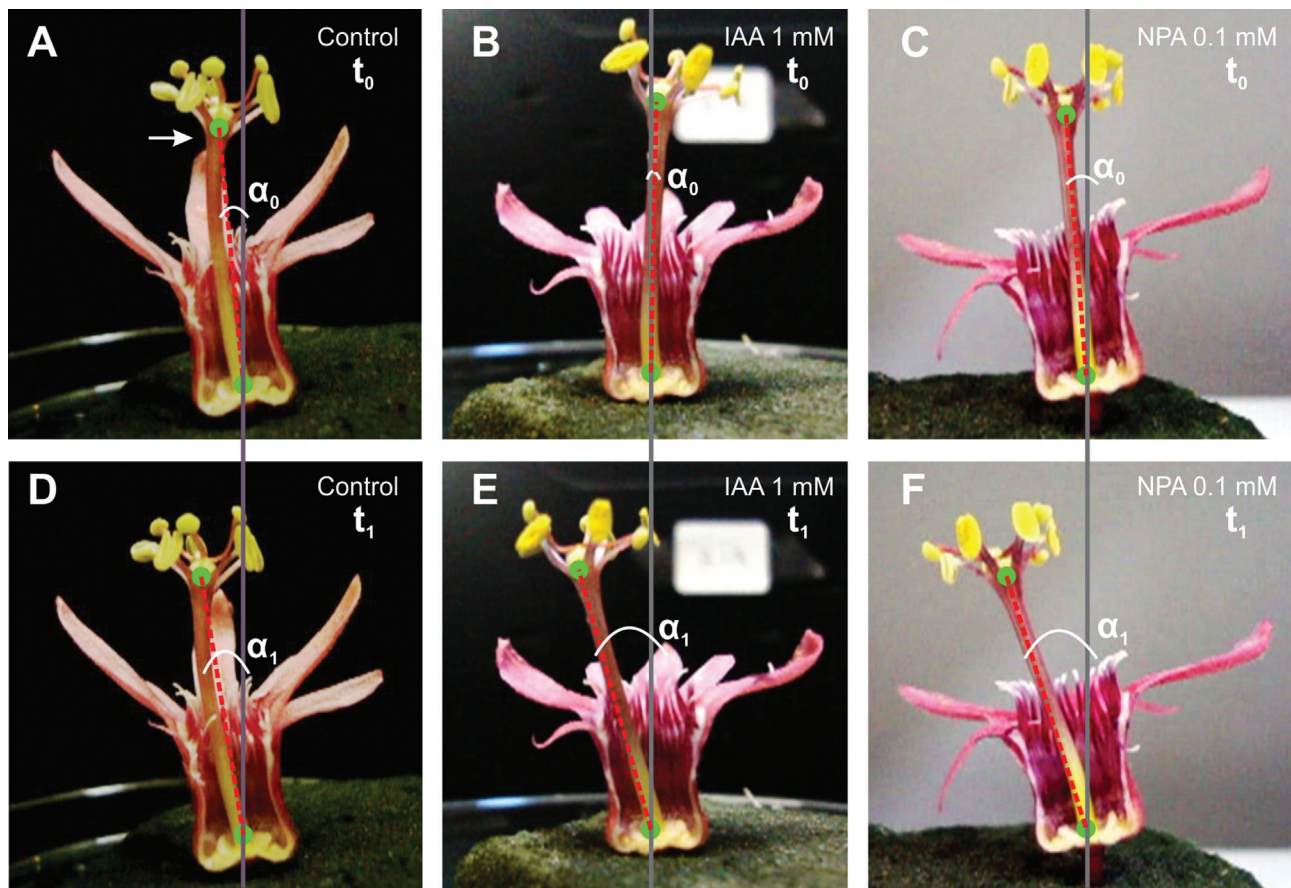

Figure 1 - Images of flowers of *P. sanguinolenta* showing the androgynophore before (A, B, C) and after (D, E, and F) mechano-stimulation, after being treated with water (control, A and D), IAA 1 mM (B and E) and NPA 0.1 mM (C and F). The images of the androgynophore before and after the movement were put exactly in the same position related to the X-axis to clearly illustrate the change in position. The movement amplitude can be measured by $\alpha_1 - \alpha_0$, where α_0 is the angle formed by the androgynophore axis before the movement (t_0) and a line traced perpendicularly to the image; and α_1 is the angle formed between the androgynophore axis after the movement (t_1) and the same perpendicular line. Note that when IAA or NPA is applied the amplitude of the movement is clearly greater than in the control.
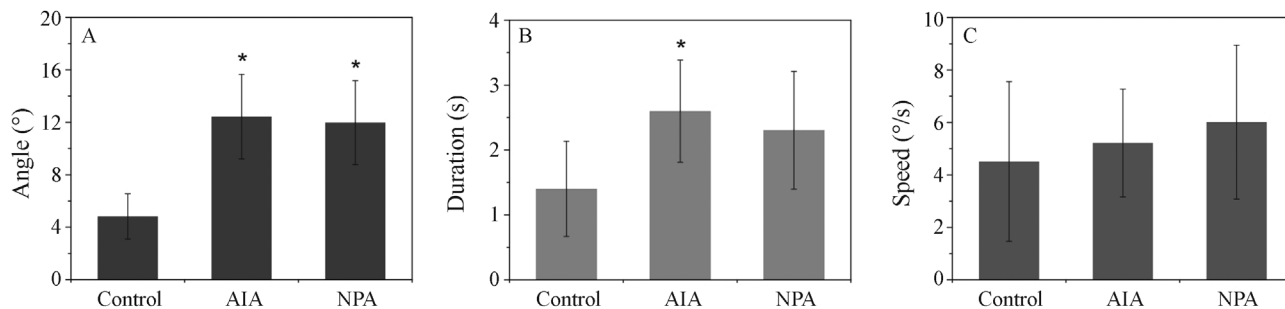
Figure 2 - Effect of the IAA and NPA treatments on the movement dynamics of the *P. sanguinolenta* androgynophore. (A) Movement amplitude measured by the angle formed between the initial state (before the movement) and the final state (after the movement) in degrees. (B) Duration of the movement in seconds. (C) Angular speed of the movement in deg/s. Data represent means ± SD. Asterisks indicate statistical differences from the control (according to Kruskal-Wallis test).

find any difference when comparing the speed of the movement in flowers treated with IAA and the water control treatment (Figure 2).

Flowers that were immersed in the auxin polar transport inhibitor NPA also showed a significant increase in the values of the angle formed between the steady state and after the movement (Figures.1 and 2). When analyzing the duration of the movement we did not find a statistical difference between the NPA treated and the water control, albeit there was a tendency to an increase in time (Figure 2). Opened flowers of *P. sanguinolenta* sprayed with the NPA test solution did not show any difference in the dynamics of the movement compared to control flowers that were not sprayed (Figure 3).

As it has been previously suggested that the hummingbird-pollinated *P. sanguinolenta* is derived in relation to other insect-pollinated *Decaloba* species (Scorza and Dornelas, 2014), we tested whether the flowers of the interspecific hybrid between *P. sanguinolenta* and the insect-pollinated *P. capsularis* inherited thigmotropic androgynophore features. We observed that both *P. capsularis* and *P. sanguinolenta* presented similar angles formed by the androgynophore trajectory, but *P.* 'capsang' showed a much wider movement when compared to the parental species (Figure 4A). While the duration of the androgynophore

movement was significantly shorter in *P. capsularis* when compared to *P. sanguinolenta*, it was even shorter in the interspecific hybrid (Figure 4B). Therefore, the speed that the *P.* 'capsang' androgynophore took to bend after the mechanical stimulus was more than twice the one observed for the parental species (Figure 4C).

Discussion

We tested whether exogenous application of auxin (IAA) or an inhibitor of its polar transport, NPA, would alter the thigmotropic movement pattern of androgynophores of *P. sanguinolenta*.

In a preliminary test we wanted to see whether spraying a solution containing NPA at 0.1 mM would generate any response on the movement. Our results showed that only spraying opened flowers was not sufficient to induce any effect on the movement, probably because the epidermis, together with the cuticle, was a barrier difficult to pass through. We did not intend to spray or treat young flower buds continuously with the "petiole-feeding" technique (Lin *et al.*, 2011; Rocha *et al.*, 2015) as this could alter the normal development of the flower organs (Benkova *et al.*, 2003).

When we adopted a treatment procedure of dipping the flower pedicels at anthesis in the treatment solutions for
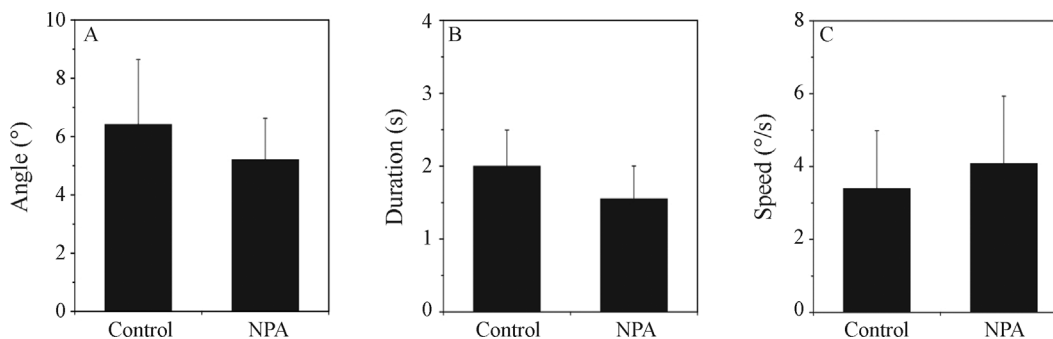
Figure 3 - Effect of spraying NPA on opened flowers. (A) Movement amplitude measured by the angle formed between the initial state (before the movement) and the final state (after the movement) in degrees. (B) Duration of the movement in seconds. (C) Angular speed of the movement in deg/s. The data shows that only spraying NPA is not sufficient to cause alteration in the androgynophore movement.
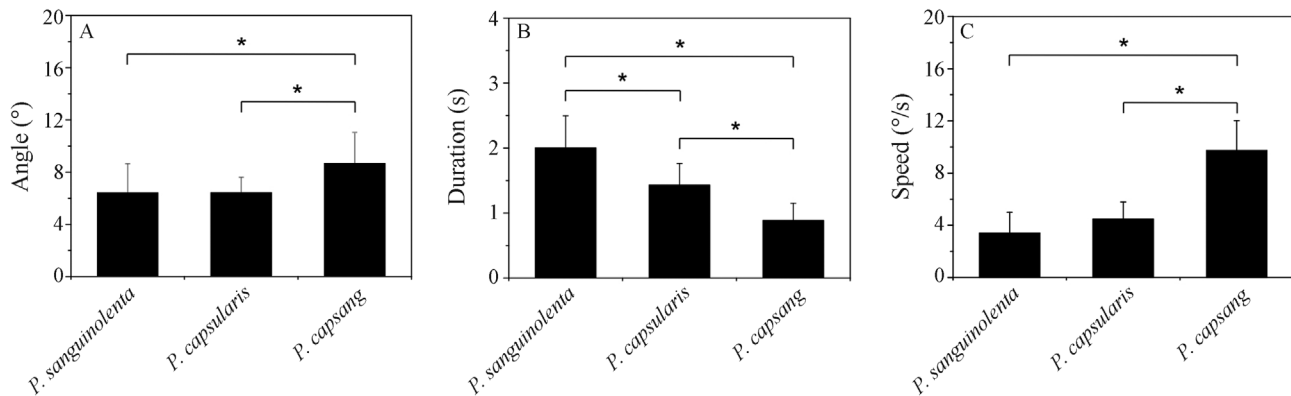
Figure 4 - Movement dynamics of the androgynophore of *P. capsularis*, *P. sanguinolenta* and their interspecific hybrid *P.* 'capsang'. (A) Movement amplitude measured by the angle formed between the initial state (before the movement) and the final state (after the movement) in degrees. (B) Duration of the movement in seconds. (C) Angular speed of the movement in deg/s. Data represent means ± SD. Asterisks indicate statistical differences between genotypes (Kruskal-Wallis test).

3 h we were able to detect effects of IAA and NPA on the androgynophore movement. We observed that flowers treated with a 1 mM IAA solution showed a significant increase in the amplitude of their androgynophore bending movement (Figures 1 and 2). This then caused an increase in the duration of the movement, as the androgynophore took more time to make the longer trajectory while the speed of the movement was unaltered when compared to the water control (Figure 2).

As we had previously shown that *Passiflora* androgynophore movement presents certain mechanistic similarities with pulvinar movements (Scorza and Dornelas, 2014; Scorza *et al.*, 2014), it is worthy of note that when IAA or 2,4-D were applied to detached leaves of *C. fasciculata*, the amplitude of the leaflet opening induced by light increased, whereas the dark-induced closure of the leaflets was inhibited (Bourbouloux *et al.*, 1992; Bonmort and Roblin, 1996). When *M. pudica* leaflets were mechanically stimulated in darkness after application of IAA, rapid closure and opening were observed, similarly to what happens in daylight (Watanabe and Sibaoka, 1983). 2,4-D had a more drastic effect and inhibited touch-induced leaflet closure in *M. pudica* (Moyen *et al.*, 2007). The mechanism by which auxins influence the pulvinar movements is the activation of H^+-ATPase proton pumps in the plasma membrane, leading to ion and water influx to the cells. This process leads to an increase in turgor pressure and the pulvinar cells are kept constantly turgid, preventing the leaflets to fold up during the dark induction period, and increasing the amplitude of the leaflet opening during the light induction. In *P. sanguinolenta* a similar effect of increased turgidity might have occurred, but the turgidity was not refractory to touch as sometimes also happened with Fabaceae thigmonastic leaflets treated with auxins, and where the androgynophore was still inclined in response to a mechanical stimulus (Samejima and Sibaoka, 1980; Moran, 2007; Volkov *et al.*, 2010a). We have already shown that the basis of the movement of *Passiflora* motile androgynophores is the swelling

and shrinking of the androgynophore epidermis and parenchyma cells (Scorza *et al.*, 2014). If the cells get more turgid it is more likely that they also have the potential to lose more water when plasmolysed after being touch-stimulated. This would explain the increase in the amplitude of the *P. sanguinolenta* androgynophore movement, as seen after IAA treatment.

Auxin is a weak organic acid that enters the cell easily through diffusion across the plasma membrane (Zazímalová *et al.*, 2014). When inside the cells, most of the auxin dissociates in the anionic form, which makes it more difficult to be transported out of the cells. Auxin efflux carrier proteins promote the transport of auxin from cell to cell in a directional manner (Benkova *et al.*, 2003). This process is commonly referred to as auxin polar transport, and it has been related to various aspects of plant development. The polar transport inhibitor NPA is probably the most effective inhibitor of auxin polar transport (Petrásèk *et al.*, 2003). NPA impairs the auxin polar efflux, but has no influence on decreasing IAA concentration or activity; on the contrary, it can increase auxin accumulation in the cells (Morris *et al.*, 2005; Petrásèk *et al.*, 2003). In our experiments, when NPA was applied to *P. sanguinolenta* flowers, a very similar effect to IAA treatments was observed, as the amplitude of the androgynophore movement was also increased.

In *Arabidopsis*, stamina are major sites of IAA accumulation during flower development (Aloni *et al.*, 2006). As well as in many other species, *Arabidopsis* petals only develop after stamina have almost fully developed. It has been shown that the high IAA production in young organs, especially in stamina, inhibits the development of other organs, such as petal elongation (Aloni *et al.*, 2006). The androgynophore column only develops at later stages of *P. sanguinolenta* development, after the stamina and the gynoecium have already developed (our observations). The androgynophore column elongates concomitantly with petals and corona filaments, which also develop later during flower bud formation, suggesting that a mechanism similar

to stamen inhibition by auxin might be involved in *P. sanguinolenta* flower development. Under the hypothesis that the tip of each floral organ is a primary site of auxin production that can induce its own development and differentiation, and sometimes inhibit the growth of neighboring organs (Aloni *et al.*, 2006), it is reasonable to assume that the androgynophore, including the column, the stigma and the gynoecium, also produce auxin, which might be transported basipetally. When NPA is applied, the auxin that might be produced by the *P. sanguinolenta* androgynophore would not be transported and, thus, accumulate at its base, generating a similar response as when exogenous IAA is applied.

In *Passiflora* there are many examples of flowers in which selective pressure has driven the evolution of novel mechanisms that impact on the reproduction and survival (Lindberg and Olesen, 2001; Aizza and Dornelas, 2011; Rocha *et al.*, 2015). In the flowers of *P. sanguinolenta*, *P. citrina*, *P. capsularis* and *P. rubra*, the motile androgynophore seems to be a novel feature that maximizes pollen deposition onto pollinators - hummingbirds in species with tubular flowers such as *P. sanguinolenta* and *P. citrina* and insects in bowl shaped flowers, such as those of *P. capsularis* and *P. rubra*. As we mentioned, the cellular basis of the movement is a subtle loss of turgor in cells at the stimulated side of the androgynophore, which is capable of turgor recovery within minutes, a mechanism that enables the organ to respond to a new stimulus, *i.e.*, other pollinators visiting the flower (Scorza and Dornelas, 2014; Scorza *et al.*, 2014). Auxins are implied in maintaining the cell turgor. Accordingly, the cells of the androgynophore in our study were sensitive to the application of this hormone, showing as a phenotypic response an increase in the amplitude of the movement, which, in turn, has a potential role in the process of pollen transfer onto pollinators. This process seems to be especially decisive on the reproduction of self-incompatible species, as *P. sanguinolenta* and *P. citrina* (Scorza and Dornelas, 2014). Taken together, these data put in evidence a probable role of auxin in modulating the fitness of these *Decaloba* species to different pollinators.

Another issue is whether the thigmotropic androgynophore features are adaptively inherited for their potential role in the evolution of flower-pollinator relationships in *Passiflora*. It has been suggested that hummingbird-pollinated *Xerogona* species, such as *P. citrina* and *P. sanguinolenta*, are derived in relation to other insect-pollinated *Decaloba* species (Milward-de-Azevedo *et al.*,, 2014). We therefore infer that the wide-moving androgynophores, characteristic of hummingbird-pollinated species (such as *P. sanguinolenta*) are derived with respect to insect-pollinated species with more 'restricted' movement (such as *P. capsularis*). As artificial interspecific hybrids, used as ornamental plants, are widely available among *Passiflora* species (Ulmer and MacDougal, 2004), we as-

sessed the thigmotropic androgynophore features of *P.* 'capsang' and its parental species, *P. capsularis* and *P. sanguinolenta*. We observed that the androgynophore movement of the hybrid is two-times faster than that of the parental species. Although we cannot discard the hypothesis that this particular phenomenon is of epigenetic nature, it seems more likely that it can be explained as a 'hybrid heterosis effect' (Birchler *et al.*, 2003). Future studies on the gene expression profiles of the parental species and the interspecific hybrid should shed light on this unresolved issue. Although the genetic nature of this heterosis remains to be determined, our results suggest that the characteristic features (such as speed) of the androgynophore movement are prone to be under evolutionary pressure. Taken together and assuming that faster androgynophore movement can provide a greater fitness to flowers that are pollinated by hummingbirds, our results suggest general mechanisms by which hummingbird-pollinated flowers can arise from insect-pollinated ancestors.

Acknowledgments

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Associate Editor: Igor Schneider