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Calcium and the generation of plant form

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SUMMARY

The involvement of mechanical signals (tension and compression) in the determination of the form of living organisms has been speculated upon for many years. These mechanical signals (both environmental and those generated within the plant itself) have significant effects on plant development and thus morphology.

Plants respond to externally applied mechanical signals (touch and wind) by an immediate elevation of cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) in stimulated cells. This response requires the movement of plant tissues to cause tension and compression. Some of the more longer-term responses to mechanical signals, e.g. *TCH* gene expression and reduction in hypocotyl growth, show a calcium-dependency.

It seems likely, therefore, that the effects of mechanical signals on plant development are mediated by the second messenger, calcium. This raises the exciting possibility that this simple ion plays a central role in the determination of plant form itself.

1. INTRODUCTION

Many plants are extremely sensitive to touch. Early work has put a value on this sensitivity with reports that some plant tendrils are sensitive to masses of 25 µg (Pfeffer 1906). The sensitivity of the tentacle of the common sundew (*Drosera rotundifolia*) was examined by Charles Darwin. By using tiny fragments of human hair he was able to obtain tentacle inflection with a mass as small as 0.82 µg (Darwin 1875). This touch response is therefore two orders of magnitude more sensitive than the touch sense of the average human. In a separate set of experiments, Darwin described the responsiveness of roots from numerous species which bend in response to touch (Darwin 1880). There are numerous other examples of less obvious touch-sensitive responses. There are reports in the literature that: (i) coleoptiles stroked with a cork rod bend to the stroked side (Stark 1921); (ii) etiolated plants stroked with sheets of paper respond as if exposed to white light (the stems are shortened and thickened (Bunning 1941; Bunning & Lempnau 1954); (iii) plant stems which are rubbed respond by synthesising ethylene and exhibit subsequent stem shortening and thickening, a phenomenon termed thigmomorphogenesis (Jaffe 1973); and (iv) stroking roots interrupts their growth for several hours (Hanson & Trewavas 1982). Pollen is also touch sensitive and pollen tubes grow in defined directions on material with repetitively spaced ridges (Hirouchi & Suda 1975).

Wind is a well-recognized morphogenic stimulus (Grace 1977). In woody plants the wind-induced rocking of the stem about the roots leads to a diversion of carbohydrate resources to induce stem thickening and lignification. The effects of wind can be mimicked by intermittent shaking to sway the plant, a phenomenon called seismo-morphogenesis (Jones & Michell 1989). Shaking also induces premature dormancy in young trees and substantial height and yield reductions in maize (Neel & Harris 1971, 1972).

In addition to acting as environmental signals, there can be no doubt that mechanical signals within plant tissues themselves are also important in determining how a plant develops. Tension and compression patterns will determine directions of cell division and organogenesis (Trewavas & Knight 1994). The mechanisms by which plant cells can sense and act on mechanical signals, whether external or internal, are likely to be the same (Trewavas & Knight 1994). This seems to be substantiated by the fact that touch induced (*TCH*) genes whose expression is elevated by environmental mechanical signals such as touch and wind (Braam & Davies 1990) also show elevated expression in tissues undergoing tension and compression in normal development (Sistrunk *et al*. 1994). As intracellular calcium has been shown to be involved in response to touch and wind (Jones & Mitchell 1989; Knight *et al*. 1991, 1992) it seems likely that it must be involved in the determination of plant form itself during normal development.

To answer questions regarding how mechanical signals mediate their effects in plants we are using molecular genetic approaches to try and understand the fundamental basis of the perception and transduction of mechanical signals in plants. We are also investigating the involvement of intracellular calcium in more detail to understand how this ubiquitous second messenger mediates responses specific to the mechanical signal transduction pathway(s) in plants.

2. MECHANICAL SIGNALS ELEVATE CYTOSOLIC-FREE CALCIUM

Aequorin is a calcium-sensitive luminescent protein from the jellyfish *Aequorea victoria*. In the jellyfish, high quantities of aequorin are found in specific cells. When

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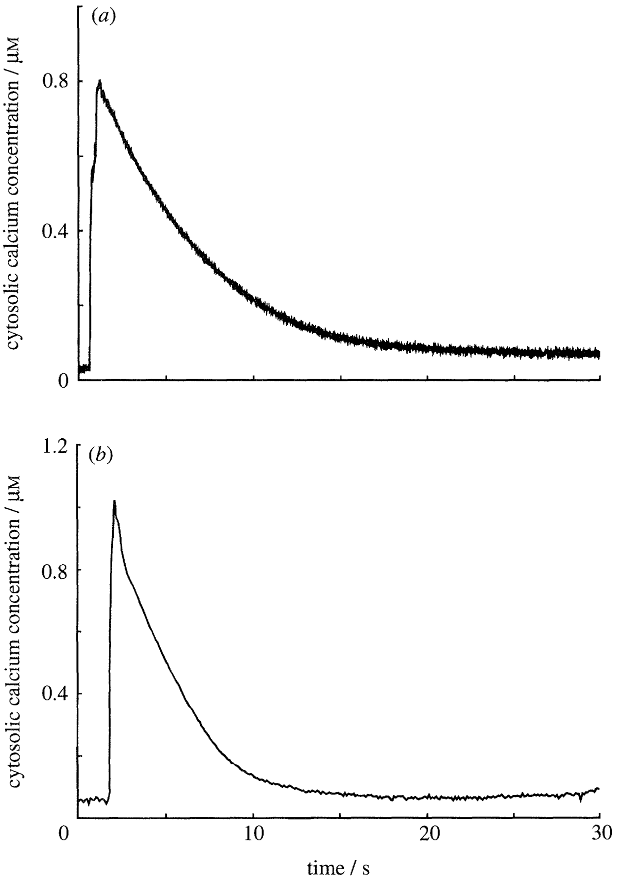

Figure 1. Effect of touch stimulation on cytosolic free calcium in *Nicotiana plumbaginifolia* (*a*) and *Arabidopsis thaliana* (*b*) both expressing apoaequorin and treated with coelenterazine. Luminescence was measured as described previously (Knight *et al.* 1991). Single plants (six day-old *Arabidopsis* or two week-old *Nicotiana*) were placed in water and an equal volume of water injected to mediate the response. Free calcium concentrations were calculated (Cobbold *et al.* 1983) and plotted against time.

touched, cytosolic free calcium rapidly elevates in these cells causing the jellyfish to luminesce. Aequorin is a 21 kDa protein composed of an apoprotein, apoaequorin, and an imidazolopyrazine luminophore, coelenterazine. On interaction with calcium (Ca^{2+}), coelenterazine is oxidized to coelenteramide and luminescent light is emitted. Calibration of emitted light against free Ca^{2+} is relatively straightforward in free solution and in single cells (Knight *et al.* 1993). We have genetically transformed tobacco (*Nicotiana plumbaginifolia*) to express apoaequorin and showed that incubation of the transformed seedlings in coelenterazine lead to reconstitution of actively-reporting aequorin (Knight *et al.* 1991). Because aequorin is a soluble protein these luminous plants have luminosity which directly reports cytosolic free calcium. These transformed plants show touch-induced elevations in cytosolic free calcium ($[Ca^{2+}]_{cyt}$). Figure 1 shows the touch response of *Nicotiana plumbaginifolia* and *Arabidopsis thaliana* expressing aequorin.

The mechanism whereby touch and wind stimuli modify plant growth and development is not understood but changes in intracellular calcium most probably transduce both stimuli. Aside from the measurements described above, EGTA and calmodulin-binding inhibitors negate rub-induced growth reductions in soybean (Jones & Mitchell 1989). Much more dramatically touch stimulation (mainly rubbing or stroking) of *Arabidopsis* massively induces the expression of five touch genes, three of which have been identified as calmodulin or calmodulin-related proteins (Braam & Davis 1990). These important data demonstrate that mechanical stimulation can specifically modify gene expression and again indicate a substantive role for $[Ca^{2+}]_{cyt}$.

One fundamental but vital question is ‘how can calcium mediate specific responses?’ Cytosolic free calcium elevations occur in response to a whole variety of signals as well as mechanical ones, for example, cold-shock, fungal elicitors, wounding and oxidative stress (Knight *et al.* 1991, 1992, 1993; Price *et al.* 1994). Somehow the plant can discriminate between these responses and effect a specific response. One source of the specificity could be the subcellular location of the calcium within the cell during signalling. The concentration of cytosolic calcium may rise only in specific regions of the cell in response to the different signals, hence activating different target proteins and processes which are distributed heterogenously in the cell. It becomes important therefore to determine specifically where the calcium is coming from to elevate cytosolic concentrations during different responses. Understanding the heterogenous distribution of calcium in different responses may resolve the problem of specificity.

For this reason we have previously investigated a range of calcium channel blockers, calmodulin-binding inhibitors and other inhibitors of calcium metabolism on wind-induced $[Ca^{2+}]_i$ changes (Knight *et al.* 1992). Inhibitory effects were only observed with ruthenium red at low concentrations. Ruthenium red is believed to inhibit mitochondrial calcium uptake or to inhibit release of Ca^{2+} from the rough endoplasmic reticulum (Denton *et al.* 1980). The site of action of ruthenium red is not understood in plant cells but it is generally assumed to modify $[Ca^{2+}]_i$ release from internal stores.

We are now approaching this problem another way, namely the measurement of calcium in specific cellular locations using targeted aequorin. This has allowed us to measure changes in endoplasmic reticulum calcium (N. J. Watkins, H. Knight, A. K. Campbell, A. J. Trewavas, M. R. Knight unpublished results) and microdomain calcium around the tonoplast membrane (H. Knight, A. J. Trewavas, M. R. Knight unpublished results) in response to mechanical signals.

3. MORPHOLOGICAL THIGMOMORPHOGENETIC MUTANTS

To isolate vital components of the mechanical signal transduction pathway(s) of plants we performed a mutant screening program using T-DNA-tagged *Arabidopsis thaliana* plants. We have screened 4900 independent families of T-DNA-tagged *Arabidopsis* (Feldmann 1991) for phenotypic mutants showing reduced growth responses to mechanical signals. Etiolated seedlings were mechanically stimulated and

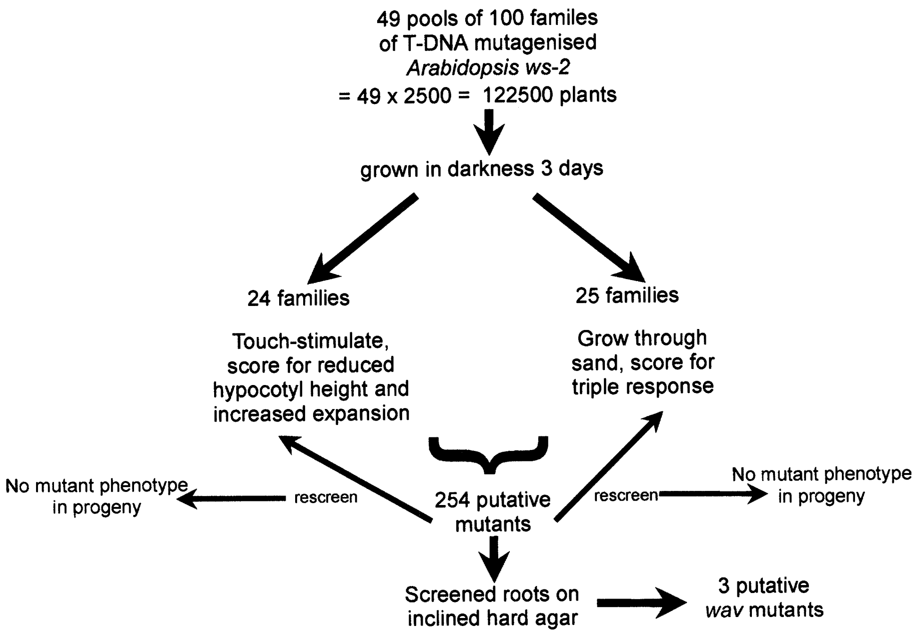

Figure 2. Screening for thigmomorphogenetic mutants of *Arabidopsis thaliana*. Plants were grown in darkness and either stimulated mechanically by hand or by growing through sand. Upon rescreening of 254 putative mutants none of these lines showed a clear mutant phenotype in the progeny. Screening of these putative mutants on inclined hard agar allowed the identification of three putative *wav* mutants (Okada *et al.* 1990).

screened for reduced hypocotyl extension/expansion and increased tightening of the hook (see figure 2). The appearance of these three morphological features together is known as the 'triple response' occurring only in response to mechanical signals. What is apparent from this type of approach is that these morphogenetic processes can be controlled by many factors other than mechanical signals and that there is a large amount of natural variation in these parameters in *Arabidopsis*. This explains the very high frequency of putative mutants none of which were *bona fide* thigmomorphogenetic mutants. Some putative thigomotropic *wav* mutants already isolated (Okada & Shimura 1990) were obtained in this screen. This illustrates a definite need for the directed and specific screening protocol such as outlined below. Only these types of approach will be successful.

4. MECHANICAL SIGNAL PERCEPTION/TRANSDUCTION MUTANTS

To develop a specific, targeted screen we decided to focus on two specific points in the pathway involved in the perception and transduction of mechanical signals, namely cytosolic calcium increases and *TCH* gene expression (see figure 3).

We have initiated a screening program in *Arabidopsis thaliana* using as a genetic background an *Arabidopsis* transgenic line which expresses apoaequorin and also the photoprotein luciferase from the firefly *Photinus pyralis* under the control of a *TCH* gene promoter. By screening for mutants using a highly-sensitive photon-counting camera (Knight *et al.* 1993) we hope to isolate mutants in parts of the pathway shown as (1) and (2) in figure 3. As well as helping to identify vital components of this pathway and hence build an understanding of the molecular basis of the perception and transduction of mechanical signals in plants, this approach will help answer the vital and fundamental question 'is calcium doing anything, and if so what?'

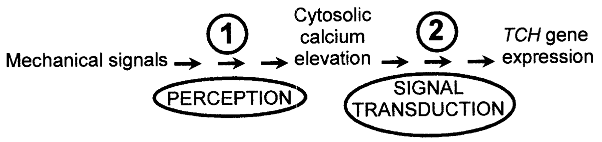

Figure 3. The mechanoperception and signal transduction pathway leading to touch gene expression.

5. CONCLUSIONS

Mechanical signals have many important effects on plant growth and development. The second messenger calcium is implicated in these responses. As mechanical signals may be important in the determination of plant morphogenesis it seems possible that calcium is involved directly in the generation of plant form. To understand how calcium can act specifically in this pathway we are investigating the organisation of intracellular calcium within plant cells during mechanical signalling. To understand the molecular basis of the perception and transduction of mechanical signals we aim to identify luminescence mutants which are impaired in touch-induced calcium signalling and *TCH* gene expression. This approach should lead to a better understanding of the exact role of calcium in this signal transduction pathway as well as bringing to light other vital components.

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