Article Addendum

Feedback control of reactive oxygen and Ca^{2+} signaling during brown algal embryogenesis

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In a recent paper in *Planta*, we combined novel confocal reflectance imaging of intracellular reactive oxygen species (ROS) with inhibition-of-growth experiments to show that ROS help to direct polarized growth in brown algal zygotes. Using confocal fluorescence imaging of intracellular Ca^{2+} distributions, we were also able to show an interaction between ROS and Ca^{2+} signaling. The modulation of intracellular Ca^{2+} signals by reactive oxygen species (ROS) is a common motif in many plant and algal systems, but our *Planta* paper is its first demonstration during early development. We explain here how our findings complement a number of recent studies on polarized growth in plant and algal systems.

In many multicellular organisms an initial asymmetric division of the zygote is a critical event during early development. The acquisition and expression of a zygotic axis of polarity ensures that the first cell division will give rise to two daughter cells with distinct cellular identities. This polarity is key to the assignation of different cell fates to different early embryonic cells and hence to the generation of correct multicellular patterning. However, the principles underlying the organization of polarity acquisition remain elusive. Different model organisms, such as the fruit fly *Drosophila melanogaster*, the flowering plant *Arabidopsis thaliana* and the brown alga *Fucus serratus*, have been used to probe different aspects of zygotic polarization and, taken together, the evidence shows that a regulatory network of genes, proteins and small signaling molecules (such as Ca^{2+} ions) act to specify the direction of polarization.

One crucial insight has been to show that axes of polarity are defined by ionic and molecular gradients. These gradients are often expressed by a number of cellular components, with protein gradients being the best studied. The most famous of these is undoubtedly

found in animals—cell specification in developing *D. melanogaster* embryos is determined by the anterior-posterior body axis.¹ This is derived from the initial axis of zygote polarity which is, in turn, defined by the famous *bicoid* transcription factor gradient set up during oögenesis in response to maternal organizers.² Similar protein gradients have also been shown to direct polarization in flowering plants. In *Arabidopsis thaliana*, for example, polarized distribution of the *PIN* auxin transporter³ helps to specify an apical-basal axis during embryogenesis,⁴ which then determines the shoot-root axis of the adult plant.

Unfortunately, fewer data are available about the role played by non-protein, small signaling molecule gradients. Many of the model organisms which allow genetic dissection of protein regulatory networks are poor models in which to perform the physiological studies needed to follow small signaling molecules. Flowering plant embryos, for example, are concealed inside ovules which supply maternal cues to polarization, making real-time visualization of small molecule gradients extremely difficult.

To circumvent this problem, small molecule gradients are often studied in brown algae.⁵ Gametes of the marine brown alga *Fucus* and *Pelvetia*, for example, are large, easy to obtain and externally fertilized, making them particularly suitable for cellular imaging studies. Furthermore, brown algal zygotes polarize in response to external cues, most notably the direction of incident light,^{6,7} allowing for precise experimental control of the direction and timing of polar axis acquisition. Such studies have revealed that zygote polarity is established in three stages; (a) the generation of asymmetries along the incident light gradient a few hours after fertilization (*axis formation*),^{6,7} (b) the stabilization of the asymmetries around 6–10 hours after fertilization (*axis fixation*)⁸ and, following this photopolarization process, (c) the separation of these asymmetries into two different cells—the rhizoid and the thallus cell (Fig. 1)—around 12–18 hours after fertilization (*germination and cell division*). This polar axis defines the orientation of zygotic growth, the plane of the first cell division, the distinct fates of the asymmetric daughter cells and the polarity of the organism, with subsequent cell divisions occurring in a highly ordered spatial and temporal pattern.⁵

What have brown algal zygotes revealed about embryogenic small molecule gradients? The most striking findings have shown that the axis of polarity in brown algal zygotes is defined, at least in part, by an intracellular Ca^{2+} gradient which can be visualized as early as 1

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hour after exposure to unilateral blue light during the photosensitive period^{9,10} and which persists through rhizoid germination and elongation.⁹

In our *Planta* paper, we decided to look at how this central Ca^{2+} gradient was maintained and to examine what controls existed to ensure its robustness. To do this, we took our cue from earlier work by ourselves and others on non-embryonic cells which display polarized growth, most notably root hairs¹¹ and pollen tubes.¹² In both of these cell types, it has been shown that Ca^{2+} influx can be stimulated by reactive oxygen species (ROS). ROS are an inescapable consequence of aerobic metabolism in eukaryotes, but may also be generated in a controlled manner by intracellular processes and have been ascribed a number of regulatory functions. One of the main sources of such regulatory ROS are the plasma membrane localized NADPH oxidases. These convert molecular oxygen to superoxide anions (O_2^-) which may, in turn, dismutate to H_2O_2 or hydroxyl radicals (OH).

Given that the ROS- Ca^{2+} link is a well-established and evolutionarily conserved signaling motif,^{13,14} it seemed sensible to investigate whether a similar ROS- Ca^{2+} interdependence might exist in *F. serratus* zygotes. The problems were largely technical; fluorescent and absorbance dyes which report ROS are 'one-shot' non-equilibrium dyes. This means that they report all the ROS production which has occurred since the dyes were introduced, rather than providing a snapshot of ROS generation during the second or so over which images were acquired. The non-equilibrium nature of the dyes meant that stringent control experiments were needed if sensible inferences were to be made about the patterns of dye intensities. We therefore used two dyes, the fluorescent H_2O_2 and OH-sensitive chloromethyl-2',7'-dichlorofluorescein (CM-DCF) and the absorbant O_2^- -sensitive nitroblue tetrazolium (NBT).¹⁵

Our results strongly suggested that the Ca^{2+} gradient was indeed interdependent on ROS generation by NADPH oxidases and supported both our earlier work in root hairs¹¹ and the work done by Nicholas Smirnov's and Victor Žárský's groups on pollen tubes.¹² Taken together, our data are consistent with a model in which ROS stimulate generation of a tip-high Ca^{2+} gradient which is amplified by positive feedback between Ca^{2+} and ROS production and then maintained by InsP_3 activity (Fig. 2).

Several questions remain. First, our paper looked briefly at a possible role for PLC and InsP_3 in supporting the intracellular Ca^{2+} gradient; our conclusion that PLC acted through its product, InsP_3 , in *F. serratus* zygotes does not agree with work done in flowering plant pollen tubes, in which PLC is believed to act through its substrate, PIP_2 .^{16,17} Whether this is a species- or cell-specific difference remains to be determined.

Second, our paper only looked at the interdependence of Ca^{2+} and ROS in zygotes during the third and final stage of polarity establishment—the germination of the rhizoid. There is some evidence that redox processes are also involved in axis formation¹⁸ and it would be interesting to know whether the ROS and Ca^{2+} signaling networks are interdependent during axis formation and fixation.

Third, and finally, the next challenge is to integrate these findings on small molecule gradients with the better characterized work on protein gradients in order to understand how different signaling and communication pathways set up a regulatory network.¹⁹ With this aim in mind, the traditional model organisms in which polarity has

Figure 1. Polarization in zygotes of the brown alga *Fucus serratus*. The spherically symmetrical zygote polarizes away from the direction of incident light (coming from the top of picture) and the resulting apical-basal axis determines the axis of the adult plant.

Figure 2. Cartoon to show possible model for the generation of polarized Ca^{2+} gradients in *Fucus serratus* zygotes. Note the reciprocal feedback between ROS and Ca^{2+} , which may act to amplify weak initial signals into longer lasting embryogenic ones.

been studied may need to be updated. What is now needed is a model with easily visualized zygotes and a tractable genome which will allow a combination of genetic and physiological approaches. Surprisingly, the best placed candidates may be the moss, *Physcomitrella patens*²⁰ and the brown alga, *Ectocarpus siliculosus*^{21,22} and we look forward to seeing what their futures hold.

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