Early Embryo Development in \textit{Fucus distichus} Is Auxin Sensitive$^1$

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Auxin and polar auxin transport have been implicated in controlling embryo development in land plants. The goal of these studies was to determine if auxin and auxin transport are also important during the earliest stages of development in embryos of the brown alga \textit{Fucus distichus}. Indole-3-acetic acid (IAA) was identified in \textit{F. distichus} embryos and mature tissues by gas chromatography-mass spectroscopy. \textit{F. distichus} embryos accumulate $[^{3}H]$IAA and an inhibitor of IAA efflux, naphthylphthalamic acid (NPA), elevates IAA accumulation, suggesting the presence of an auxin efflux protein complex similar to that found in land plants. \textit{F. distichus} embryos normally develop with a single unbranched rhizoid, but growth on IAA leads to formation of multiple rhizoids and growth on NPA leads to formation of embryos with branched rhizoids, at concentrations that are active in auxin accumulation assays. The effects of IAA and NPA are complete before 6 h after fertilization (AF), which is before rhizoid germination and cell division. The maximal effects of IAA and NPA are between 3.5 and 5 h AF and 4 and 5.5 h AF, respectively. Although, the location of the planes of cell division was significantly altered in NPA- and IAA-treated embryos, these abnormal divisions occurred after abnormal rhizoid initiation and branching was observed. The results of this study suggest that auxin acts in the formation of apical basal patterns in \textit{F. distichus} embryo development.

The basic body organization of plants and algae is established during embryogenesis, with the apical basal pattern resulting from an initial asymmetric cell division (for review, see Kropf et al., 1999; Jurgens, 2001). This first cell division leads to apical and basal daughter cells that are precursors to the shoot meristem and root meristem, respectively, in land plants and thallus and rhizoid tissues in algae, respectively. The directional cues that control the polarity of this first cell division are not yet clear in higher plants, although maternal factors have been suggested to orient the embryo axis (Jurgens, 2001). In contrast, in embryos of brown algae, environmental gradients, with light being the best characterized, control the polarity of the apical basal pattern (Belanger and Quatrano, 2000). In higher plants and algae, similarities in the mechanism by which the first asymmetric cell division is established are becoming apparent. In Arabidopsis, the product of the \textit{GNOM} gene is required for stable fixation of the apical basal axis (Mayer et al., 1993). The \textit{GNOM} gene encodes a brefeldin A (BFA)-sensitive guanine nucleotide exchange factor for small GTP-binding proteins, which function in membrane trafficking (Steinmann et al., 1999). In Arabidopsis, both the \textit{gnom} mutation and BFA treatment led to alterations in membrane protein localization, including PIN1, a putative auxin efflux carrier protein (Steinmann et al., 1999; Geldner et al., 2001). In embryos of brown algae, treatment with BFA leads to alterations in the orientation of the first cell division and to formation of embryos with multiple or branched rhizoid or basal cells (Shaw and Quatrano, 1996; for review, see Belanger and Quatrano, 2000). These results implicate vesicle secretion in early events in formation of cell polarity in land plants and algae embryos.

The plant hormone auxin may also play a role in embryo development (for review, see Geldner et al., 2000; Soutar and Lindsay, 2000). Several mutants with altered auxin responses have embryo development defects, including \textit{axr6}, \textit{bdl} (bodenlos), and \textit{mp} (monopterous; Berleth and Jurgens, 1993; Hamann et al., 1999; Hobbie et al., 2000). The mutations in the \textit{MP} and \textit{BDL} genes alter the division plane of the apical daughter cell and affect both the central and basal cell lineages (Berleth and Jurgens, 1993; Hamann et al., 1999). Because the mutations in \textit{AXR6}, \textit{BDL}, and \textit{MP} genes give rise to seedlings with no primary root, these genes appear to be required for organizing embryonic root formation, but are not necessary for postembryonic root formation (for review, see Jurgens, 2001). The nature of the proteins encoded by these genes so far indicates a role for...

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auxin-regulated gene expression in these development processes (for review, see Geldner et al., 2000).

Additional studies have suggested that embryo development may be directly tied to the ability to properly transport auxin. Treatment of embryos with inhibitors of indole-3-acetic acid (IAA) efflux led to the development of altered shapes of embryos in carrot (*Daucus carota*; Schiavone and Cooke, 1987; Schiavone, 1988), *Ipomoea batatas* (Chee and Cantliffe, 1989), mustard (*Sinapis alba*; Liu et al., 1993), wheat (*Triticum aestivum*; Fischer and Neuhaus, 1996), and *Brassica juncea* (Hadfi et al., 1998). The altered embryo structures that result from these treatments resemble the defects found in the *gnom* mutant, described above (Steinmann et al., 1999) and in plants with a mutation in the *AtPIN1* gene (Liu et al., 1993; Hadfi et al., 1998), which is predicted to encode an auxin efflux carrier (Gälweiler et al., 1998). PIN1 is normally asymmetrically localized to one plane of the plasma membrane (Gälweiler et al., 1998) and this localization is believed to control the directionality of auxin transport (for review, see Muday and DeLong, 2001). Finally, mutations in the *GNOM* gene or treatments with BFA also led to embryos with a mislocalization of PIN1 (Steinmann et al., 1999). Therefore, these results suggest interdependence between auxin transport and the targeted vesicle secretion that plays an important role in early events in embryo development.

To examine the role of auxin during the formation of the apical basal axis, *Fucus distichus* embryos provide an excellent system. *F. distichus* gametes are released into seawater and fertilization and development occur in solution, facilitating the examination of large populations of synchronized embryos (for review, see Belanger and Quatrano, 2000). A number of events that occur before the first asymmetric cell division have been identified, and these studies provide an excellent framework for understanding formation of developmental polarity (for review, see Kropf et al., 1999). Because targeted secretion, actin filaments, and ionic gradients have all been implicated in polarity formation in these embryos (for review, see Belanger and Quatrano, 2000), it should also be possible to examine the relationship between auxin and these processes in the context of early development.

In the present study, the role of auxin and polar auxin transport in the initial stages of *F. distichus* embryo development was examined. The presence of IAA in *F. distichus* was verified by gas chromatography (GC)-mass spectroscopy (MS) analysis of extracts from *F. distichus* tissue. The ability of *F. distichus* to transport IAA, and the activity of auxin efflux carriers and influx carriers, were examined. Altered embryo developmental patterns were induced by treatment with either auxin or auxin transport inhibitors, with these compounds active in the first 6 h after fertilization (AF), which is before the first cell division. Together, these experiments suggest that auxin plays a role in the formation of the apical basal pattern of these embryos.

**RESULTS**

*F. distichus* Contains Free IAA at Concentrations Comparable with Higher Plants

Extracts of both zygotes and mature tissues of *F. distichus* were prepared, IAA was purified, and GC-MS was used to demonstrate the presence of IAA and to quantify the IAA concentration. An extract from the fruiting tips of *F. distichus* was subjected to GC-MS. A total ion chromatograph (TIC) of a peak eluting from the GC with a retention time of 5.593 to 5.600 min is shown in Figure 1. The peaks at 189 and 130 mass-to-charge ratio (m/z) result from intact methylated IAA and the predominant fragmentation product, respectively. Additional fragments of the endogenous IAA are an unmarked peak at m/z 77 and a peak at 103. Ions at m/z 136 and 195 are the quinolinium ion and molecular ion from the [13C]-IAA internal standard. This fragmentation pattern of a sample with this retention time on GC is conclusive demonstration of the presence of IAA in these extracts.

Free IAA concentrations measured from extracts of mature fruiting tips and zygotes using GC-selected ion monitoring-MS are reported in Table I. The amounts of IAA were determined relative to the [13C]-IAA internal standard, using single ion chromatograms, and are compared with IAA measurements from the literature for other plants. The concentration in fruiting tips was in the range previously reported for tobacco (*Nicotiana tabacum*) leaves (Chen et al., 1988) and that reported for Arabidopsis cotyledons (Zhao et al., 2001). The free IAA concentration...
in embryos changes during development, with a range between 5 and 31 ng g fresh weight$^{-1}$ for carrot embryos (Michalczuk et al., 1992), and in wheat embryos, a range from 8 to 90 ng g fresh weight$^{-1}$ (Fischer-Iglesias et al., 2001). The free IAA concentration obtained for F. distichus zygotes is similar to the lower end of both of these ranges. The calculated IAA concentration may be an underestimation of the concentration, though, because the F. distichus embryos were removed from artificial seawater (ASW) for these analyses and it is possible that some of the ASW was retained with the embryos, thereby causing an overestimation of the weight and an underestimation of the free IAA concentration.

One concern that has been previously raised about measurement of IAA in algal samples is the possibility that the presence of IAA is due to contaminating microorganisms (Evans and Trewavas, 1991; Cooke et al., 2002). This possibility cannot be eliminated in the reported measurements of free IAA in thallus tissues collected from the ocean. This is much less of an issue with F. distichus zygotes, though. During preparation, F. distichus zygotes were filtered to remove thallus tissue and any associated microorganisms and then allowed to settle briefly under conditions that would select against contaminating microbes. In addition, other samples of zygotes were recovered in the presence of antibiotics and IAA molecules, rather than being mediated by an IAA uptake protein.

### Accumulation of [3H]IAA in F. distichus Embryos

To determine if IAA accumulation into F. distichus zygotes is carrier mediated, the ability of unlabeled IAA to prevent [3H]IAA accumulation was tested. If the uptake of IAA is carrier mediated, then increasing concentrations of unlabeled IAA should reduce the amount of radiolabeled IAA accumulation and the total IAA accumulation should be saturable. The amount of total IAA accumulation, using the [3H]IAA as a tracer to estimate the total IAA accumulation, is plotted as a function of unlabeled IAA added and is shown in Figure 2. There is a linear relationship between the amount of total IAA accumulated and the amount of cold IAA added. In contrast, in corn (Zea mays) coleoptile segments and tobacco cells, auxin concentrations of 100 μM saturate IAA accumulation (Sussman and Goldsmith, 1981; Imhoff et al., 2000). This result suggests that movement of IAA into F. distichus zygotes is due to passive diffusion of the protonated and hydrophobic IAA molecule, rather than being mediated by an IAA uptake protein.

### IAA and NPA Alter Embryo Development

The effects of IAA and the auxin transport inhibitor NPA on embryo development were examined by culturing embryos in ASW in the presence and absence of 50 μM of these compounds on horizontal slides in the dark, as shown in Figure 3. In Figure 3A,

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**Table I. Measurement of free IAA in F. distichus and land plants**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Free IAA Concentration (ng g fresh wt$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F. distichus fruiting tips</td>
<td>9.3 ± 1.1</td>
</tr>
<tr>
<td>F. distichus zygotes</td>
<td>2.9 ± 1.3</td>
</tr>
<tr>
<td>Carrot embryos</td>
<td>5–31$^{a}$</td>
</tr>
<tr>
<td>Tobacco leaves</td>
<td>5–15$^{b}$</td>
</tr>
<tr>
<td>Arabidopsis cotyledons</td>
<td>2.3$^{c}$</td>
</tr>
</tbody>
</table>


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**Table II. [3H]-IAA accumulation in F. distichus zygotes**

<table>
<thead>
<tr>
<th>NPA (μM)</th>
<th>[3H]-IAA Accumulated (fmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.8 ± 0.4</td>
</tr>
<tr>
<td>5</td>
<td>4.5 ± 0.7</td>
</tr>
<tr>
<td>10</td>
<td>6.1 ± 0.4$^{a}$</td>
</tr>
<tr>
<td>20</td>
<td>7.3 ± 0.8$^{b}$</td>
</tr>
<tr>
<td>50</td>
<td>7.6 ± 0.4$^{b}$</td>
</tr>
<tr>
<td>100</td>
<td>6.4 ± 0.2$^{b}$</td>
</tr>
</tbody>
</table>

$^{a}$ The reported values are the average and SE of a representative experiment with six replicates. $^{b}$ Indicates alterations in IAA accumulation that are significantly different from the controls with no added NPA as judged by students t test with $P < 0.02$. 

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**Naphthylphthalamic Acid (NPA) Causes Accumulation of [3H]IAA in F. distichus Embryos**

To determine if F. distichus zygotes have the ability to move IAA across membranes, and whether an IAA efflux carrier controls auxin movement with similarities to those in land plants, auxin accumulation assays were performed. F. distichus zygotes (2 h AF) were incubated for 1 h in the presence of 25 nM [3H]IAA along with a range of NPA concentrations. The [3H]IAA not associated with the zygotes was removed by filtration and the [3H]IAA accumulated within the zygotes was quantified. In land plants, NPA reduces IAA efflux (Rubery, 1990). In F. distichus zygotes, NPA also appears to block efflux, thereby increasing the accumulation of [3H]IAA, as shown in Table II. The reduction in IAA accumulation at the highest dose of NPA is often seen in other transport assays using Arabidopsis and zucchini (S.R. Brady and G.K. Muday, unpublished data), although the explanation for these bell-shaped dose response curves is unknown. The magnitude and concentration dependence of the effect by NPA is comparable with that found in tissue segments, tissue culture cells, or membrane vesicles derived from many green plants (Sabater and Rubery, 1987; Muday et al., 1995; Delbarre et al., 1996; Cooke et al., 2002).
A representative embryo with a single unbranched rhizoid that was cultured in untreated ASW is shown. Embryos grown on IAA predominantly developed multiple rhizoids, often with four or more randomly distributed rhizoids, as shown in Figure 3B. Growth on NPA led to embryos with branched rhizoids (Fig. 3C). Although most F. distichus rhizoids will ultimately form branches, these normally occur much later in development and in cells considerably further from the thallus cell. If IAA and NPA treatments are performed in the light, the magnitude of these developmental alterations are significantly reduced (data not shown), which is consistent with a previous report in which IAA had no significant effects and an IAA efflux inhibitor had only slight effects on F. distichus embryo development in the light (Torrey and Galun, 1970). Because F. distichus embryos normally are released and undergo the earliest stages of development in the light, the physiological relevance of these developmental effects of exogenous IAA and NPA should be considered. There is experimental evidence that in mosses, light suppresses auxin signals. Cryptochrome-disrupted or auxin-treated Physcomitrella patens have increased branching patterns and the cryptochrome disruptants have higher sensitivity to exogenous auxin (Imaizumi et al., 2002). Therefore, blue light, acting through cryptochrome, reduces the auxin response (Imaizumi et al., 2002) much as light reduces the branching effects of auxin on F. distichus embryos reported here. In addition, NPA and IAA do perturb F. distichus development in the light as well, but in a different way. Treatments with IAA and auxin efflux inhibitors will reduce the percentage of embryos that are polarized in response to unilateral light (S. Basu and G.K. Muday, unpublished data).

The effects of NPA and IAA, and the combination of these two compounds, on embryo development are summarized for three separate experiments using 50 μM of each compound in Table III. The reported values are for 48 h of treatment, although the results for 12 and 24 h were very similar (data not shown). IAA caused a greater than 10-fold increase in the number of embryos with multiple rhizoids. NPA also led to a slight 1.5-fold increase in multiple rhizoids, although this increase was not statistically significant (P = 0.17). When both NPA and IAA were added together, the number of embryos with multiple rhizoids was similar to when IAA was added alone.

Although IAA did not affect the percentage of embryos with branched rhizoids, NPA treatment resulted in branched rhizoids in greater than 50% of the embryos. This is a statistically significant increase over the DMSO control that leads to an increase of between 2- and 3-fold in the number of embryos with...
branched rhizoids. The magnitude of the effect is at the lower end of that range in this summary due to the unusually large number of branched rhizoids in the controls, as compared with Figures 4 through 6. When NPA and IAA were added together, IAA prevented the increased number of branched embryos induced by NPA.

To verify that the effects of IAA and NPA were not simply due to the weak acid character of these compounds, additional experiments were performed with benzoic acid (BA) as a control for nonspecific weak acid effects. In the presence of 50 μM BA, 85% of the embryos developed normally as shown in Figure 4, with a single unbranched rhizoid, which is similar to ASW controls. In the presence of either NPA or another auxin transport inhibitor, TIBA, at the same concentrations, the number of embryos with branched or multiple rhizoids increased. The P value of the Student’s t test comparing the frequency of altered embryo development in controls (BA treatments) with the NPA treatments was P = 0.0026 and with the TIBA treatments was P = 0.0033, indicating that the increases in altered developmental patterns due to both treatments were significant. Also, the effect of the solvents in which auxin and auxin transport inhibitors were dissolved were examined. In Table III, controls contain similar concentrations of ethanol or DMSO as in the treatments with IAA or NPA, indicating that the solvents lead to significantly fewer embryos with altered rhizoid formation.

### Table III. Effect of IAA and NPA on *F. distichus* developmental patterns

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Multiple Rhizoids (%)</th>
<th>Branched Rhizoids (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol control</td>
<td>6 ± 2</td>
<td>21 ± 4</td>
</tr>
<tr>
<td>IAA</td>
<td>75 ± 3[^c^]</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>Dimethyl sulfoxide (DMSO) control</td>
<td>8 ± 1</td>
<td>26 ± 1</td>
</tr>
<tr>
<td>NPA</td>
<td>12 ± 4</td>
<td>56 ± 5[^c^]</td>
</tr>
<tr>
<td>Ethanol/DMSO control</td>
<td>5 ± 0</td>
<td>16 ± 2</td>
</tr>
<tr>
<td>IAA and NPA</td>
<td>72 ± 7[^c^]</td>
<td>14 ± 4</td>
</tr>
</tbody>
</table>

[^a^] Each compound was added at 50 μM and the ethanol and/or DMSO in the controls were at equivalent concentrations to the IAA or NPA treatments, respectively.

[^b^] The reported values are the average and ± of three separate experiments, each containing 200 embryos per treatment.

[^c^] The samples that were statistically different from the controls as judged by student’s t test are indicated (P < 0.01).

**NPA and IAA Alter *F. distichus* Embryo Development in a Dose-Dependent Fashion**

The numbers of embryos with altered rhizoid formation and branching in response to a range of IAA

![Figure 4](#)

**Figure 4.** NPA and triiodobenzoic acid (TIBA) cause abnormal embryo development in the dark. The percentage of *F. distichus* embryos with branched and/or multiple rhizoids are reported in response to treatment with BA, NPA, and TIBA at 50 μM after 48 h. The average and ± of three separate experiments are reported. Asterisk, P < 0.005 as judged by Student’s t test, showing a statistical difference from BA-treated control.

![Figure 5](#)

**Figure 5.** IAA and NPA led to dose-dependent changes in *F. distichus* embryo development. The percentage of the total number of embryos with multiple or branched rhizoids after culture for 48 h in ASW containing either NPA or IAA is plotted as a function of concentration of added compound. Results from a representative experiment are shown that was repeated three times.
and NPA concentrations were quantified and results from a representative experiment are reported in Figure 5. IAA led to a dose-dependent and more than 5-fold increase in the number of rhizoids. The auxin transport inhibitor, NPA, caused a dose-dependent increase in the number of branched rhizoids, with greater than a 3-fold increase. The concentration of IAA at which 50% of the embryos had multiple rhizoids was 31.6 μM and 50% of embryos were branched at 49.5 μM NPA. Again, NPA also increased the embryos with multiple rhizoids, but with complex concentration dependence and with effects on 25% or fewer embryos. The NPA concentrations that alter development were similar to those that affected IAA accumulation. Maximal effects on IAA accumulation were observed at 50 μM NPA, whereas the IC₅₀ for formation of branched rhizoids was 49.5 μM NPA. In addition, the IAA and auxin transport inhibitor concentrations that caused F. distichus embryo alterations are at the high end of the range of concentrations found to alter land plant embryo patterns, in which IAA concentrations in the range of 1 to 40 μM were effective (Liu et al., 1993; Fischer and Neuhaus, 1996; Hadfi et al., 1998).

IAA and NPA Alter Position of Planes of Division in F. distichus Embryos

Because NPA and IAA exert their effect early in development, the possibility that these compounds alter the orientation of division planes was examined. F. distichus embryos were treated with IAA and NPA for durations of 12, 24, or 48 h, beginning 2 h AF, and then stained with fluorescein diacetate (FDA), which facilitates the localization of division planes (Henderson, 1998), as shown in Figure 7. The control embryos grown in ASW for the 12 h treatment period had germinated, but cell division had not yet occurred (Fig. 7A). By 24 h, most embryos had undergone one division, resulting in formation of thallus and rhizoid cells (Fig. 7B), whereas at 48 h, multiple divisions
have occurred, resulting in single unbranched rhizoids (Fig. 7C). Treatment with IAA (Fig. 7, D–F) resulted in germination of multiple rhizoids before the first cell division, as well as a random orientation of division planes and development of a rhizoid from several of the thallus cell divisions. Most of the embryos treated with NPA also had altered orientation of division planes but with less serious alterations, but again these division plane alterations followed the formation of branched rhizoids (Fig. 7, G–I). Solvent controls treated with 0.5% (v/v) ethanol and DMSO were similar to those treated with ASW (data not shown). These results indicate that alterations in rhizoid initiation and branching precede cell division.

DISCUSSION

The goal of these experiments was to explore the role of auxin and auxin transport in the development of apical basal polarity. *F. distichus* is an ideal system for these experiments because it is possible to obtain thousands of synchronized embryos in which the ability of the auxin, IAA, and the auxin transport inhibitor, NPA, to alter development can be quantitatively examined. Because these brown algae are relatively distantly related to land plants, it was critical to first determine if these algae contain IAA and whether there is evidence for carrier-mediated influx and efflux of IAA.

IAA was detected in both zygotes and mature tissues of *F. distichus* with an abundance of free IAA that is similar to, but slightly lower than that found in land plants (Chen et al., 1988; Michalczuk et al., 1992; Zhao et al., 2001) and other green plants (Cooke et al., 2002). The identification of IAA by GC-MS is consistent with the previous identification of compounds with auxin activity in *F. distichus* using bioassays (du Buy and Olson, 1937; Overbeek, 1940). In addition, auxin movements in *F. distichus* embryos were measured by examination of [3H]IAA accumulation into embryos. IAA accumulation was found to increase upon treatment with NPA. Because NPA acts specifically at the site of auxin efflux (Rubery, 1990), these results are consistent with an auxin efflux carrier with similar pharmacology to those found in land plants. The inability of excess unlabeled IAA to reduce [3H]IAA accumulation into embryos suggests that IAA uptake is not mediated by a protein carrier in *F. distichus* zygotes, but may rather occur through passive diffusion of a protonated and hydrophobic IAA molecule. Although both uptake and efflux proteins are known to function in land plants, the possibility that one set of carriers is more highly conserved evolutionarily has not been experimentally tested.

A number of investigators have surveyed diverse groups within the plant kingdom to test for the presence of auxin metabolism and transport (Rubery, 1986; Sztein et al., 2000; for review, see Cooke et al., 2002). IAA has been demonstrated convincingly to be present using GC-MS in a number of species including Charophytes, mosses, and liverworts, but with differences in IAA metabolism between plant groups (Sztein et al., 1995, 1999, 2000). In addition, there is evidence for the presence of polar auxin transport in bryophytes, mosses, and the green alga (for review, see Cooke et al., 2002). The brown algae, of which *F. distichus* is a member, are evolutionarily quite distant from the other algae and land plants. The brown algae are part of Kingdom Phaeophyta, which are in the Stramenophiles clade, which is distinct from the clade including Plantae (Tudge, 2000; Cooke et al., 2002). Therefore, it is somewhat surprising that there are apparently similar mechanisms of auxin efflux used in brown algae and land plants, but this finding does suggest the universal importance of auxin transport in plants and algae.
Because *F. distichus* zygotes contain free IAA from the earliest stages AF and have evidence for IAA efflux carrier-mediated transport, then it was reasonable to examine the role of auxin in *F. distichus* embryo development. Treatment of embryos with either exogenous IAA or the IAA efflux inhibitor, NPA, led to altered rhizoid formation, but with two different characteristics. Multiple rhizoids were evident upon treatment with IAA, with four or more randomly distributed rhizoids often formed. When both IAA and NPA were added simultaneously, the number of embryos with multiple rhizoids matched that found with IAA alone. This is consistent with a dominant effect of IAA on this process and with external IAA concentrations controlling rhizoid initiation. Similarly, a previous report indicates that culturing of brown algae in the presence of exogenous auxin for 40 d caused increased number of holdfasts, which is the structure that develops from rhizoids (Davidson, 1950). Treatment with NPA led to high numbers of embryos with branched rhizoids, although IAA-treated embryos did not exhibit this phenotype. In fact, treatment with IAA in addition to NPA reduced the number of branched rhizoids to the levels in untreated embryos. This reversal of the NPA effect by IAA is consistent with NPA acting to alter IAA distribution.

The effects of NPA and IAA on *F. distichus* embryo development can only be partially compared with the effects of these compounds reported for embryos of land plants. Although a number of treatments were performed on zygotic embryos, the effects of the treatments were reported on embryos cultured for 1 or 2 weeks on these compounds (Fischer and Neuhaus, 1996; Fischer et al., 1997; Hadfi et al., 1998). The significantly longer exposure to added compounds and the absence of information on the effects on the earliest divisions make it difficult to compare these previous studies with those reported here. Although higher concentrations of auxin and auxin transport inhibitors were required to alter *F. distichus* embryos, the ability to expose the embryos for less than 1 h and get nearly maximal effects indicates that the embryos are quite sensitive to these compounds. The best comparison of auxin effects on the first cell divisions can be made by examination of Arabidopsis mutants. The *bdl* (*bodenlos*) and *axr6* mutants have altered auxin sensitivity and show increases in number of embryos with an alteration in the apical basal pattern (Hamann et al., 1999). In *bdl*, although 6% of the embryos have a defect normally, this mutation causes that to be elevated to 18% of the embryos (Hamann et al., 1999).

The timing of the effects of IAA and NPA on embryo development was carefully examined. Treatments for 0.5 h indicate that the maximal effects of IAA are between 3.5 and 5 h and for NPA between 4 and 5.5 h AF. It is possible that the developmental significance in the delay in NPA action relative to IAA is due to the effect of IAA on rhizoid initiation and the effect of NPA on later events leading to rhizoid branching. Therefore, it is clear that IAA and NPA exert their effect very early in the developmental sequence. In these single-celled zygotes, IAA movements are detectable across the *F. distichus* plasma membrane, yet there are clearly no intercellular IAA movements. Therefore, it becomes important to consider the function of IAA efflux at this early developmental stage.

First, if polar IAA movement is integrally linked to the polarity of each cell, then it is conceivable that the polarity of IAA efflux is established simultaneously with the development of polarity. Although [3H]IAA accumulation assays cannot provide insight on whether IAA is polarly transported, they do demonstrate the NPA-regulated movement of IAA in these zygotes, consistent with very early auxin transport activity. These results suggest one additional conclusion, which is that the appropriate distribution of IAA is required for establishment of polarity because inhibition of IAA efflux or excess IAA led to alterations in the formation of apical basal pattern. The simplest explanation for this result is that the IAA efflux carrier complex is part of the membrane protein complex that forms at the site of rhizoid initiation to mark this location for growth and that a gradient of IAA across the rhizoid tip is essential to reinforce the signal for rhizoid outgrowth. Consistent with this idea is the evidence that local cell wall loosening precedes rhizoid germination in brown algae (Hable and Krofp, 1998). In land plants, cell wall loosening is required for growth and is stimulated by auxin (Rayle and Cleland, 1992). In addition, the timing of the cell wall loosening in *Pelvetia compressa* rhizoids, occurring predominantly at 4 to 6 h AF (Hable and Krofp, 1998), is later than the period of NPA and IAA sensitivity. The multiple rhizoids that initiate in the presence of excess external IAA are consistent with a delocalization of the rhizoid initiation signal, perhaps mediated by multiple cell wall loosening events. The excess rhizoid branching in the presence of NPA might then arise from a reduction in the strength of the external IAA signal that reinforces the initiation point resulting in a weaker initiation signal and several local rhizoid initiation events.

A second important question about these developmental alterations is the role of cell division in this process. In *F. distichus* embryos, the formation of apical basal polarity precedes the first cell division, in which rhizoid germination is evident by 12 to 14 h, yet division does not occur until approximately 24 h AF. In the case of NPA and IAA, it is clear that the effects of these compounds are on rhizoid formation and that altered cell division is the result, rather than the cause, of these alterations. In support of this conclusion is the evidence that NPA and IAA complete their action in less than 6 h AF (Fig. 6), well before the first cell division, and that germination of
multiple or branched rhizoids is documented in cells that have not yet undergone division (Fig. 7). This conclusion is also supported by Shaw and Quatrano (1996), who conclude from their studies with BFA that the cytoskeleton/cell wall complex formed at the site of rhizoid outgrowth directs the rhizoid formation independent of cell division.

It is critical to consider the role of IAA in the context of the other signaling events that control embryo development in brown algae. Formation of an F-actin patch has been observed at the site of fertilization of 
P. compressa embryos (Hable and Kropf, 2000) and this patch will disassemble and reform at the ultimate site of rhizoid inhibition, if the position of signals that control initiation are shifted (Alessa and Kropf, 1999). The detection of an actin patch that predicts the site of rhizoid initiation has the best temporal correlation to the window in which NPA and IAA exert their effect, usually being first evident at about 3 h AP in 
P. compressa (Alessa and Kropf, 1999). In addition, the actin cytoskeleton has been implicated in the localization of IAA efflux carriers through attachment to one protein that is part of the IAA efflux carrier (Butler et al., 1998; Hu et al., 2000; for review, see Mudad, 2000) and as tracks to deliver vesicles containing auxin transporters (Geldner et al., 2001; for review, see Mudad and Murphy, 2002). If elevated IAA concentration on the outside of the zygote is required to reinforce the site of rhizoid initiation, association of efflux carriers with the newly formed actin patch may provide the mechanism to localize the IAA efflux carrier complex, just as it has been suggested to localize channels that move calcium into the embryo and facilitate calcium entry at the rhizoid tip (Robinson et al., 1999; Belanger and Quatrano, 2000). Finally, consistent with this idea, treatment of land plants with cytochalasin D leads to both a randomization of PIN1, a putative IAA efflux carrier, and to reduction in polar IAA transport (Butler et al., 1998; Kropf, 1999). In addition, the connections between auxin and targeted vesicle secretion in the formation of apical basal polarity in embryos should be considered. In both land plants and 


BFA, the GNOM ADP-ribosylation factor-guanine nucleotide exchange factor protein, also led to embryo defects that are very similar to those resulting from IAA efflux inhibitor treatment (Steinmann et al., 1999). The GNOM mutation and BFA treatment also led to randomization of the PIN1 protein, which is a putative IAA efflux carrier (Steinmann et al., 1999; Geldner et al., 2001). BFA treatments also block IAA efflux from tissue culture cells (for review, see Morris, 2000), suggesting parallel changes in PIN1 localization and transport capacity. These results then demonstrate that inhibition of vesicle transport and IAA efflux cause similar developmental defects and that vesicle transport is necessary for IAA transport. In addition, temporal and spatial control of vesicle movement to specific membranes may be essential for establishment of auxin transport polarity, consistent with the dynamic nature of BFA-dependent PIN1 membrane localization (Geldner et al., 2001).

Yet, it may also be possible that auxin transport is more deeply tied to vesicle secretion. Geldner et al. (2001) have demonstrated that the IAA efflux inhibitor, TIBA, can perturb the localization of PIN1. Specifically, they report that TIBA will prevent the BFA-induced randomization of PIN1 or the relocalization of PIN1 after BFA removal (Geldner et al., 2001). Although TIBA does not affect PIN1 localization directly, it interferes with the effect of BFA and, even more significantly, prevents the restoration of PIN1 localization after BFA is removed. Understanding of this aspect of the interaction between IAA efflux and vesicle targeting awaits further experimentation (for review, see Mudad and Murphy, 2002), yet provides an alternative explanation for the similar embryo defects induced by NPA and BFA.

In conclusion, this report contains evidence supporting a role for auxin and auxin transport in the early events of 


Materials and Methods

Chemicals

[^3H]IAA was purchased from Amersham International (Arlington Heights, IL; 25.0 Ci mmol^-1). N-1-NPA was purchased from Chemical Services (West Chester, PA). 2,3,5-TIBA and all other chemicals were purchased from Sigma (St. Louis).

Fucus distichus Zygote Isolation

Reproductive fronds (receptacles) of sporophytes of 


In developing embryos of 


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TIBA, or BA at 50
/H9262
moved and the zygotes were resuspended in ASW containing IAA, NPA, immediately after shedding into Multiscreen HA filtration plates (Millipore, values.

Examination of the Temporal Sensitivity to NPA and 

Salem (NC), and stored in the dark at 4°C for 2 to 3 weeks. The gametes were released into ASW (450 mM NaCl, 10 mM KCl, 9 mM CaCl2, 30 mM MgCl2, 16 mM MgSO4, and 10 mM TES buffer, pH 7.5) and fertilization and develop-

partment of zygotes were performed at 14°C ± 1°C in constant illumination with cool-white fluorescent lights at 60 µmol m−2 s−1, as described previ-

ously (Quatrano, 1980). Fertilization is defined as 15 min after reproductive

fronds are placed in the light. Experiments were initiated at 2 h AF, unless

noted otherwise. The germination rate for all samples independent of treat-

ments was greater than 85% unless indicated otherwise.

Identification of IAA and Quantification of Free
IAA Concentrations

Free IAA was purified and quantified using the procedure of Chen et al. (1988) in the lab of Jerry D. Cohen (U.S. Department of Agriculture, Belts-

ville, MD). Between 5 and 15 g fresh weight of F. distichus fruiting tips and 50 to 200 mg of F. distichus zygotes (2 h AF) were used for isolation of free IAA. Tissue was frozen in liquid nitrogen and ground in a mortar and pestle with glass beads and IAA extraction buffer (65% [v/v] isopropanol and 35% [w/v] 0.2 m imidazole buffer, pH 7.7). [13C]IAA (20 ng) was used as an internal standard and 50,000 dpm of [3H]IAA was used as a radiotracer. Extraction was for 1 h at 4°C and the extract was subjected to centrifugation at 10,000g for 10 min. The supernatant fluid was then analyzed for free IAA. IAA was purified by an amino column, with several organic washes and eluted in methanol with 5% (v/v) acetic acid. After concentration, the sample was purified by HPLC, methylated using ethereal diazomethane, and then analyzed by GC-MS. The GC-MS was used for a TIC of F. distichus fruiting tip extracts for identification of IAA and for selected ion monitoring to quantify the free IAA concentrations in extracts from both F. distichus fruiting tips and zygotes.

[3H]IAA Accumulation

Zygotes in ASW at a density of 40,000 zygotes mL−1 were transferred immediately after shedding into Multiscreen HA filtration plates (Millipore, Bedford, MA). The zygotes were incubated in 25 nm [3H]IAA in a total assay volume of 200 µL, in the absence and presence of NPA with IAA at the indicated concentrations in ASW with MES substituted for TES, pH 5.5, at 14°C ± 1°C in the lighted incubator for 1 h. [3H]IAA accumulation was then determined by filtration to remove the excess [3H]IAA and washing with 200 µL of ASW-MES. The filters were punched out and soaked for 30 min in 250 µL of water and 2.5 mL of scintillation fluid was added. Samples were counted in a LS 6500 scintillation counter (Beckman Instruments, Fullerton, CA) for 2 min. Background was determined by addition of 25 nm [3H]IAA to zygotes immediately before filtration and was subtracted from all the values.

Effects of IAA and Auxin Transport Inhibitors on Embryo Development

Aliquots of F. distichus zygotes (2.5 h AF) in ASW at a density of 2,000 zygotes mL−1 were placed in tubes and allowed to settle. ASW was re-

moved and the zygotes were resuspended in ASW containing IAA, NPA, TIBA, or BA at 50 µM or a range of concentrations. Slides were coated with poly-L-lys and baked at 100°C for 15 min and cooled to room temperature. For each treatment, 60 µL of the solution containing zygotes was transferred to a glass slide under normal laboratory lighting conditions, and placed horizontally in a petri dish containing the same solution. The petri dish was

placed in the incubator at 14°C in the dark for 48 h. Images were obtained with an Axioplan microscope (Zeiss, Jena, Germany) equipped with an Orca cooled CCD camera (Hamamatsu Corporation, Bridgewater, NJ). The percentage of embryos with more than one rhizoid or with branched rhizoids was determined after 48 h under the dissecting scope.

Examination of the Temporal Sensitivity to NPA and IAA Treatment

To determine when NPA and IAA exert their maximal effect, zygotes at 2 h AF were treated for 1 h in the dark in ASW containing 50 µM of either

IAA or NPA and then slides were transferred to untreated ASW for the remainder of 48 h, after which branched and multiple rhizoids were quan-

tified. At least 200 embryos per treatment were scored and each experiment was replicated at least three times. The average and se of these three experiments are provided.

In a second experiment, embryos were placed in ASW and then trans-

ferred to ASW containing 50 µM of either NPA or IAA for 30 min. At the end of this 30-min treatment, slides were transferred back into untreated ASW after a brief rinsing with ASW. The numbers of branched embryos in the presence of NPA or embryos with multiple rhizoids were determined and the numbers of altered embryos are reported as a function of the time of the treatment.

Effect of IAA and NPA on Orientation of Division
Planes in F. distichus Embryos

Microscope slides were coated with poly-L-lys as described above. At 2 h AF, three aliquots of 100 µL of ASW with F. distichus zygotes at a density of 2,000 zygotes mL−1 were added on the coated portion of the slides, allowed to fix for 1 min, and immediately placed in ASW supplemented with NPA or TIBA at 50 µM under normal laboratory lighting conditions. The slides containing embryos were then placed at 14°C in the dark for either 24 h or 48 h. ASW containing DMSO or ethanol was used for controls at the same concentrations as in the treatments (0.5% [v/v]). After the treatments, the embryos were stained with FDA at 1 µg mL−1 according to Henderson (1998) for 7 min in the dark and fluorescent images were captured by a Zeiss Axioplan microscope equipped with a Hamamatsu Orca cooled CCD camera.

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