Morphogenesis of *Ginkgo biloba* leaf development

Ellen Zelinsky

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Abstract

*Ginkgo biloba* is a tree native to China, and the only extant species in its class. It is also known as a fossil species because it has changed very little from the fossils of *Ginkgo* found dating back to the Jurassic period. This makes it a very interesting topic of study, because it allows us to see what kinds of mechanisms terrestrial plants used for reproduction and growth before flowering plants evolved. In this study, we looked at the development of the Gingko leaf in terms of cell division patterns and gene expression of GbC4HDZ02, a gene involved in epidermal development.

Introduction

*Ginkgo biloba* is a very old species of tree, most widely known for the medicinal benefits of its seeds. However not much research has been done on the rest of the tree. Hara wrote about the unique way that a *Ginkgo* leaf develops in his paper from 1980, and since then no other major research has been done on this topic. In Hara's paper, he describes how the primordium of a *Ginkgo* leaf growing from the shoot apical meristem (SAM) forms two perpendicular bifurcations, and then expands and unfolds into the mature leaf (Figures 1 and 2). In this process, the epithelial layer formed on the inner part of the primordium becomes the adaxial side of the mature leaf when it unfolds. However, Hara did not specify how the bifurcations are formed, and that was the goal of this research.
Figure 1. The development of a *Ginkgo* primordia: A showing the earliest stage, B showing the first bifurcation, C the second, and D the third (Hara 1980).

Figure 2. A more detailed view of the different stages of splitting in a *Ginkgo* primordia. Stage A again being the youngest, and stage F being the cross-section of a mature leaf. The dots on each figure represent the xylem and phloem (Hara 1980).

Several aspects are involved in researching the methods of bifurcation. First, to look at which cells are dividing in early stage primordia, and then the expression of epithelial genes. The gene we are looking at is the second *Ginkgo biloba* class IV homeodomain leucine zipper gene, hereafter referred to as C4HDZ02. This gene is part of a class of genes known to play a role in the regulation of epidermal development (Zalewski et al. 2013).

Figure 3. Part of the gene tree showing the relationship between C4HDZ02 and *Arabidopsis thaliana* genes ML1 and PDF2. The majority of our knowledge concerning the function of C4HDZ02 comes from its close relation to these *A. thaliana* genes.
Materials and Methods

Materials

Primordia were extracted from the shoot apical meristem of Ginkgo seedlings, which were growing outside of the lab. The extraction process was then done using a microscope, tweezers, and a razor blade. If not immediately used, the primordia were then either fixed in FAA (Formaldehyde, Acetic acid, Alcohol), or snap frozen and stored at -80°C.

EdU Test

The EdU test was performed using the Click-iT EdU Alexa Fluor 488 Imaging Kit (C10337). Samples were soaked in EdU solution under growth conditions for either 3, 4, 4.5, or 5 hours to determine the best conditions for the test. Ages of primordia also varied from primordia with just one bifurcation, to older primordia which had finished splitting already (Figures 1 and 2). Additionally, some primordia were soaked in EdU solution while still on the SAM, while others were removed completely from the SAM.

RNA Extraction and RT-PCR

Extraction of RNA was done using the PureLink Plant RNA Reagent kit (small-scale isolation) followed by two-step RT-PCR. The RT-PCR was done using SuperScript III kit with oligo dT primers and the PCR mixture used ExTaq and gene specific primers to get cDNA of C4HDZ02. The primers used were GbC4HDZ02 Forward (5'-AATGGGTATGCGAAGGTGAC-3') and GbC4HDZ02 Reverse (5'-GAGCCAAGCGACAAATTAGC-3'). The cDNA was then sequenced by subcloning of amplified cDNA using AMPureXP magnetic bead kit. The PCR mixture for ligation was made using the quickTaq kit and electrophoresis of 8 of the resulting colonies was run. Three of these colonies were then selected to use for sequencing, using the BigDye kit. Sequence results were analyzed using CLC Main Workbench and compared to sequence data from Zalewski et al. (2013).

C4HDZ02 Expression Levels
RNA extraction was performed again, this time using two sets of samples. One composed of primordia with 1 or 2 bifurcations, and the other composed of more mature primordia that had finished splitting already (Figure 4). The RNA extraction for the small primordia was performed using the RNeasy micro kit (Qiagen) modified for plant tissue. The extraction for the large primordia was performed using PureLink Plant RNA Reagent and RNeasy mini kit (Qiagen) modified for plant tissue. The control for the RT-PCR was a set of tubes for each sample containing water in the place of the reverse transcriptase (-RT). Primers of *Ginkgo* 18S (Shen et al. 2005) were used as an additional control. New primers for C4HDZ02 were used to have a shorter cDNA: GbC4HDZ02 short Forward (5'-ACAGTGCACGAATCAACGAC-3'), and GbC4HDZ02 short Reverse (5'-TCAGTGCACGAATGAGTCC-3'). The RT-PCR was performed using the SuperScript III protocol again, this time using random hexamers. The PCR was set up with two primers, C4HDZ02 and 18S, each with four samples: (1) small primordia -RT, (2) large primordia -RT, (3) small primordia +RT and (4) large primordia +RT. Then each of those samples was given three tubes, labelled A, B, and C. The samples were denatured at 94°C for 2 minutes, followed by 40 cycles of amplification for C4HDZ02 (94°C for 30s, 45°C for 30s, 68°C for 1 min) and 35 cycles for 18S (94°C for 30s, 55°C for 30s, 68°C for 1 min). For C4HDZ02 samples, A tubes were removed after 30 cycles, B tubes after 35, and C tubes after 40. For 18S samples, A tubes were removed after 25 cycles, B tubes after 30, and C tubes after 35. The PCR products were separated on a 1.5% agarose gel stained with ethidium bromide.

![Figure 4. Example of the two sizes of primordia used for testing expression levels of C4HDZ02.](image)
Results and Discussion

EdU Test

EdU tests were performed and the S-phase cells in primordia were observed. The best conditions for the EdU were found to be between 4.5 and 5 hours, which had the fewest number of primordia with no signals. The signals were also the clearest on the primordia which had been completely removed from the SAM. If the bifurcations were formed by an increase in cell division along the bifurcation axis, then there would be an increased number of signals right around the splits in the primordia observed. The majority of the primordia did not have any signals directly along the split, but had many signals scattered throughout the primordium. Some of the older primordia showed a pattern of groups of dividing cells along the edge of the leaf between the bifurcations. This could be an indication that cell division actually increases on either side of the split, rather than on the split, so that tissue is built up along either side of the axis thereby creating a fissure. On the other hand, the lack of signals could just mean that cells die to form the gap that becomes the bifurcation instead of dividing.

Figure 5. Images of primordia under fluorescent light (left), and under normal light (right). The top two images are a primordium with two bifurcations, one of which is not visible in this picture. The lower
two images are a more mature primordium which has finished splitting.

**C4HDZ02 Sequencing**

Bacteria colonies were put through electrophoresis using a 1% agarose gel with ethidium bromide. All of the colonies except for the fourth one appeared to be of the expected length for bacteria with our plasmid, and so colonies 6, 7, 8 were selected to use in sequencing. The sequences obtained were compared to sequence data for the C4HDZ02 gene using CLC Main Workbench (Figure 7).

**Figure 6.** Gel electrophoresis of the eight bacteria plasmids from subcloning. The last three bands represent colonies 6, 7, and 8, the three used for sequencing. The band representing colony 4 shows that that colony did not have my plasmid.

**Figure 7.** Sequence data for two of the colonies compared to sequence data of C4HDZ01, C4HDZ02, and C4HDZ03.

The sequence results showed that colonies 6 and 8 had the correct sequence for C4HDZ02, and that the
sequences do not match the two other forms of the gene. This confirms that C4HDZ02 is in fact expressed in the primordia. The next step was to look at the amount of expression in primordia, to see if there was any difference in expression between early stage primordia, with one or two bifurcations, and late stage primordia which have finished splitting.

**C4HDZ02 Expression Levels**

The purpose of testing the difference in expression levels between the two sizes of primordia was to try and see if C4HDZ02 is expressed higher in one over the other. If the gene is more highly expressed in smaller primordia, then it would mean that C4HDZ02 is involved in the creation of bifurcations. The image on the left in Figure 8 shows the C4HDZ02 results. Some samples seemed to have gotten contaminated, as seen by the longer bands in the C4HDZ02 results, and the bands in the -RT sections of the gel, on the right side. The shorter bands in the C4HDZ02 results are the correct length for the shorter primers used for this. On the gel, there is no band for 3B, and a very faint band for 4B, which means that 4B has higher expression than 3B. Additionally, 4C has higher expression than 3C. These two together show that the larger primordia samples, which were the number 4 groups, have higher expression of C4HDZ02 than the small primordia samples.

![Figure 8. Gel electrophoresis showing the results of the gene expression test. The samples are ordered as follows starting from the left: 3A, 4A, 3B, 4B, 3C, 4C, 1A, 2A, 1B, 2B, 1C, 2C. The image on the](image-url)
right is the 18S control group, with samples ordered the same way.

These results were not what was expected. There was still a difference in expression levels as expected, but instead of the smaller primordia having higher expression, the larger primordia did. There are a few possible explanations for this occurrence. One of these explanations could be that C4HDZ02 is simply not involved in the formation of the bifurcations, but another explanation could be that larger primordia have more complex structures which demand higher expression of genes involved in epithelial development. In other words, the expression of C4HDZ02 does go up during splitting, but goes up even more in larger primordia due to the demands of the tissues. One last explanation is that the internal tissues of the primordium cause suppression of expression in some ways, so total expression levels seem higher in larger primordia, which have a higher surface area to volume ratio. In order to figure out which of these explanations is the right one, in-situ hybridization has to be performed. This will identify the location in the primordia in which C4HDZ02 is being expressed.

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References

