Telomeres, telomerase and plant development

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Telomeres are nucleoprotein structures that cap the ends of linear eukaryotic chromosomes. Almost all plant telomeres contain a characteristic heptanucleotide, TTTAGGG, repeated in a tandem array that extends for several kilobases. The length of the telomeric-DNA tract is dynamic, and subject to striking expansions and contractions that correlate with cellular differentiation. Telomeres are synthesized and maintained by the action of telomerase, a ribonucleoprotein with reverse transcriptase activity. Telomerase expression profiles mirror changes in telomere length, with the highest enzyme levels and telomere lengths associated with cells that have unlimited capacity for proliferation. The excitement of current research using *Arabidopsis* is that it is the first opportunity for studying the regulation of telomeres and telomerase in a genetically tractable, multicellular organism.

Eukaryotes ensure the integrity of their linear chromosomes by sealing the ends with specialized nucleoprotein structures known as telomeres. The unique properties of telomeres were recognized by Barbara McClintock 60 years ago. Using dicentric chromosomes that broke between the centrosomes during anaphase, McClintock found that the sister chromatids fused together at their new ends following replication, recreating a dicentric chromosome that was susceptible to breakage at the next division[1,2]. In gametophytes and endosperm, this 'breakage–fusion bridge cycle' persisted for many cell divisions; in the embryo, however, chromosomes became stabilized. This stability was interpreted to be the result of 'healing' or the acquisition of normal telomere function. Research since then has confirmed that telomeres provide a protective cap that imparts stability to chromosomes by protecting against end-to-end fusion, recombination and exonucleolytic degradation.

The importance of the telomere is underscored by recent studies demonstrating that the maintenance of telomeres on human chromosomes strongly correlates with the capacity for cellular proliferation and carcinogenesis[3]. This review focuses on plant telomeres, their synthesis by the enzyme telomerase and how telomerase regulation may be related to growth and development.

Telomeric DNA

Molecular analysis of telomeres began in the late 1970s when it was discovered that the most distal portion of *Tetrahymena* chromosomes consisted of tandem arrays of the sequence 5'-TTGGGG-3' (Ref. 4). Subsequent work revealed that telomere structure is highly conserved and that almost all eukaryotic telomeres are composed of simple G-rich repeats. The first telomere of a multicellular eukaryote was cloned from *Arabidopsis*[5]. A genomic library enriched for sequences at the ends of high molecular weight DNA was screened for clones that hybridized to exonuclease-sensitive regions of the genome. One such clone was recovered, and the telomeric nature of its sequence was confirmed by showing that it detected an RFLP linked to telotrisomy in a population segregating for this trait. The clone also hybridized to exonuclease-sensitive regions of the maize and human genomes. The *Arabidopsis* telomere clone contained 53 copies of the heptanucleotide 5'-TTTAGGG-3'. Subsequent studies confirmed that maize telomeres contain the same repeat[6], and that human telomeres have the related hexanucleotide TTAGGG (Ref. 7).

The basic repeat seen in *Arabidopsis* telomeres has been detected at the ends of chromosomes in almost every plant examined. Usually the detection has been based on *in situ* hybridizations[8], but in some cases telomeric DNA has been cloned and sequenced. In addition to *Arabidopsis* and maize, telomeric DNA has been sequenced from wheat, barley and tomato[9]. The tomato telomere sequence was determined by genomic sequencing to be TT[T/A]AGGG (Ref. 10). The degenerate third position was initially thought unusual, but variants of the basic repeat are now known to be present in the telomeres of all plants examined.

Unlike the situation in higher plants, where almost all telomeres are composed of TTTAGGG repeats, telomeres in green algae are more variable. Although telomeres of *Chlorella vulgaris* contain the TTTAGGG repeat[11], telomeres of *Chlamydomonas reinhardtii* contain the related octamer TTTTAGGG (Ref. 12). Chlorarachinophytes are protists that have captured and maintained a green algal endosymbiont. The genome of the endosymbiont has been greatly reduced, and it currently contains only three chromosomes with a total of about 400 kb of DNA. The telomeres of this unusual alga use TCTAGGG as the repeating motif[13].

Plants of the onion family (*Alliaceae*) apparently lack the TTTAGGG-type telomeres, as this repeat cannot be detected by *in situ* hybridization or Southern blotting[8]. Ribosomal RNA genes have been detected at the ends of some onion chromosomes by *in situ* hybridization[14], but the resolution of this technique cannot eliminate the possibility of additional sequences distal to the rDNA loci. Whatever the structure of telomeres in the *Alliaceae*, alternatives to the *Arabidopsis*-type telomere certainly exist in plants. *Drosophila* chromosomes are also capped by telomeres that do not fit the 'standard' pattern, consisting of retrotransposons that actively accumulate at chromosome ends[15].

Telomere-binding proteins

In addition to the simple nucleotide repeats, telomeres also contain specific non-nucleosomal proteins. Two classes of proteins that bind telomeric DNA have been identified[16]. One class, found in ciliated protozoa, forms a protective cap on the chromosome. A second class, found in other organisms, coats the double-stranded portion of the telomere. In addition to their protective function, telomere-binding proteins may regulate the length of the telomeric DNA tract[17]. Evidence is now accumulating that the double-strand-specific telomere proteins, Rap1 in yeast and TRF1 in mammals, regulate telomere length by a mechanism that 'counts' the number of telomere protein molecules on the telomere[17]. When the protein number is low, telomerase can extend the telomere

tract. The TRF1 and Rap1 proteins contain regions with high homology to the DNA-binding domain of the Myb oncoproteins[18], and these function as transcription factors. This Myb-like motif in telomere-binding proteins has been dubbed the 'telobox'[19].

Using a gel mobility shift assay, Zentgraf detected a protein from *Arabidopsis* nuclear extracts that binds both single- and double-stranded telomeric DNA[20]. The protein has greater affinity for double-stranded DNA, and for the G-rich single strand than the C-rich single strand. Denaturing-gel electrophoresis of proteins in the mobility-shifted complexes showed a single prominent band of 65 kDa. Interestingly, proteins harboring the 'telobox' motif have been found as expressed sequence tags (ESTs) from *Arabidopsis* and rice[19]. Whether the Myb-related proteins encoded by the plant ESTs actually bind to telomeres and whether they correspond to the activity identified by electrophoretic mobility shift assays[20] remain to be established.

Telomere synthesis via telomerase

The predominant mechanism for generating and sustaining telomeres on chromosome ends is through the action of telomerase, an unusual reverse transcriptase. Telomerase catalyzes the synthesis of telomere repeats, replenishing terminal DNA sequences on the ends of chromosomes that are not effectively duplicated by the conventional DNA replication machinery[21]. Telomerase is a ribonucleoprotein, and both RNA and protein subunits are essential for enzyme activity. The telomerase RNA subunit has been characterized in a variety of different organisms, including ciliates, yeasts and mammals[21]. Each RNA harbors a short sequence complementary to the telomere that provides a template for the synthesis of G-rich telomeric repeats. The protein composition of the telomerase particle is less clear. Two proteins from *Tetrahymena*, p80 and p95, co-purified with telomerase activity and were subsequently shown to bind specifically to the telomerase RNA and to telomeric DNA[22]. Mammalian homologs for p80 have been reported[23,24]. In addition to p80 and p95, a third protein, p123/EST2, was found in humans, fission and budding yeasts and the ciliate *Euplotes*[25-27]. The p123/EST2 protein appears to be the catalytic subunit of telomerase, because it carries classical reverse transcriptase motifs that are essential for telomere synthesis both *in vitro* and *in vivo*[25]. Although the complete composition of an enzymatically active telomerase particle is currently unknown, it seems likely that telomerase exists as a holoenzyme, made up of one RNA subunit, multiple protein subunits and auxiliary factors that modify its behavior[28]. Telomerase activity has been detected in a wide variety of plant species, but none of the subunits for any plant telomerase has been identified.

A model for telomere synthesis via telomerase is depicted in Fig. 1. Telomerase binds the single-stranded, G-rich overhang on the telomere and then copies the templating domain from the RNA to extend the telomere by the addition of nucleotides to its 3'-terminus. Once the end of the templating domain is reached, the nascent 3'-end of the DNA is translocated back to the beginning of the RNA template for another round of nucleotide synthesis.

Telomerase activity can be assayed by two methods. The standard method relies on detecting the addition of discrete, telomere-repeat units to a G-rich primer that resembles the natural terminus of the chromosome[29]. This method results in a ladder of products in which the most prominent bands in the ladder are separated by the length of the repeat (seven nucleotides in plants). The second method, the telomere repeat amplification protocol (TRAP) is similar, but much more sensitive, because it uses PCR to amplify telomerase products[30]. Both methods have been used to detect telomerase activity in plants.
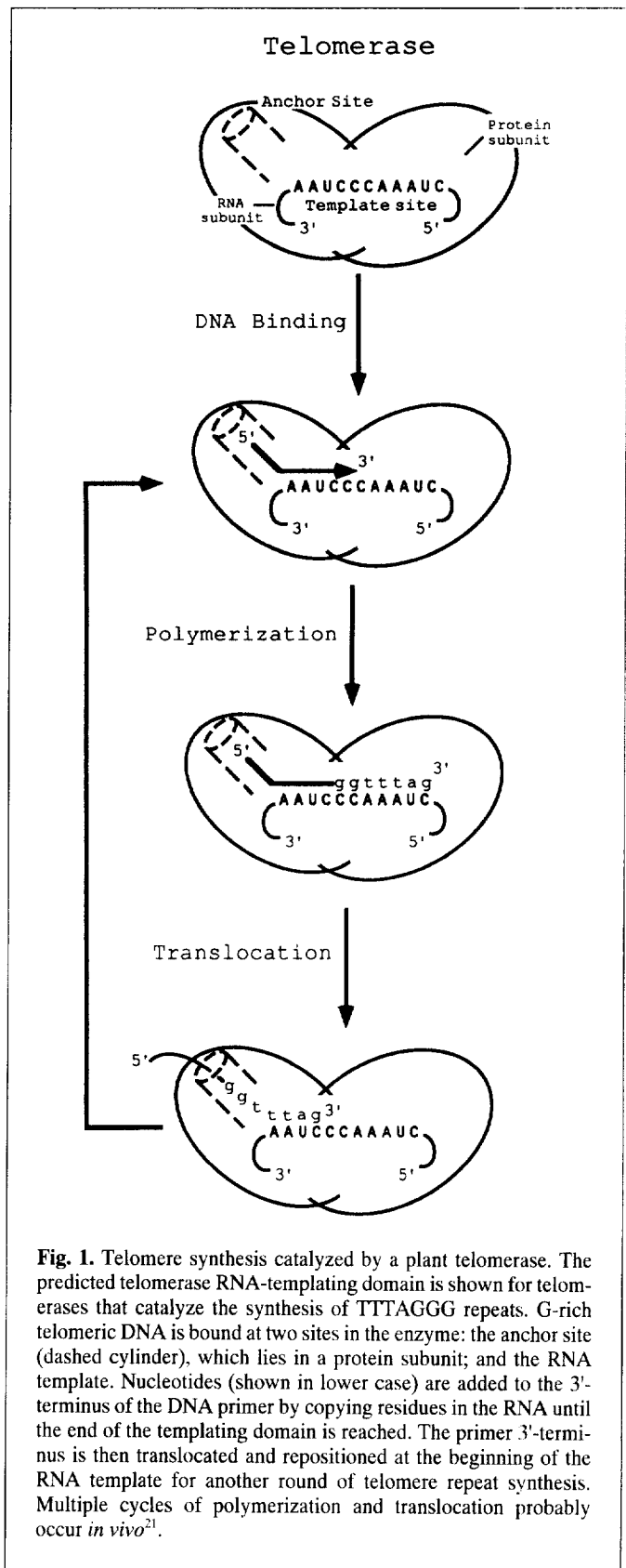

Fig. 1. Telomere synthesis catalyzed by a plant telomerase. The predicted telomerase RNA-templating domain is shown for telomerases that catalyze the synthesis of TTTAGGG repeats. G-rich telomeric DNA is bound at two sites in the enzyme: the anchor site (dashed cylinder), which lies in a protein subunit; and the RNA template. Nucleotides (shown in lower case) are added to the 3'-terminus of the DNA primer by copying residues in the RNA until the end of the templating domain is reached. The primer 3'-terminus is then translocated and repositioned at the beginning of the RNA template for another round of telomere repeat synthesis. Multiple cycles of polymerization and translocation probably occur *in vivo*[21].

De novo *telomere formation via telomerase*

Telomerase has highest affinity for G-rich telomeric DNA, but the enzyme can initiate synthesis on non-telomeric DNA that lacks complementarity to the telomerase RNA template[31]. *De novo* telomere formation provides an explanation for the 'healing' of

— Older embryo,
30 kb

— Immature embryo,
80 kb

— Mature inflorescence
spike, 20 kb

— Young inflorescence
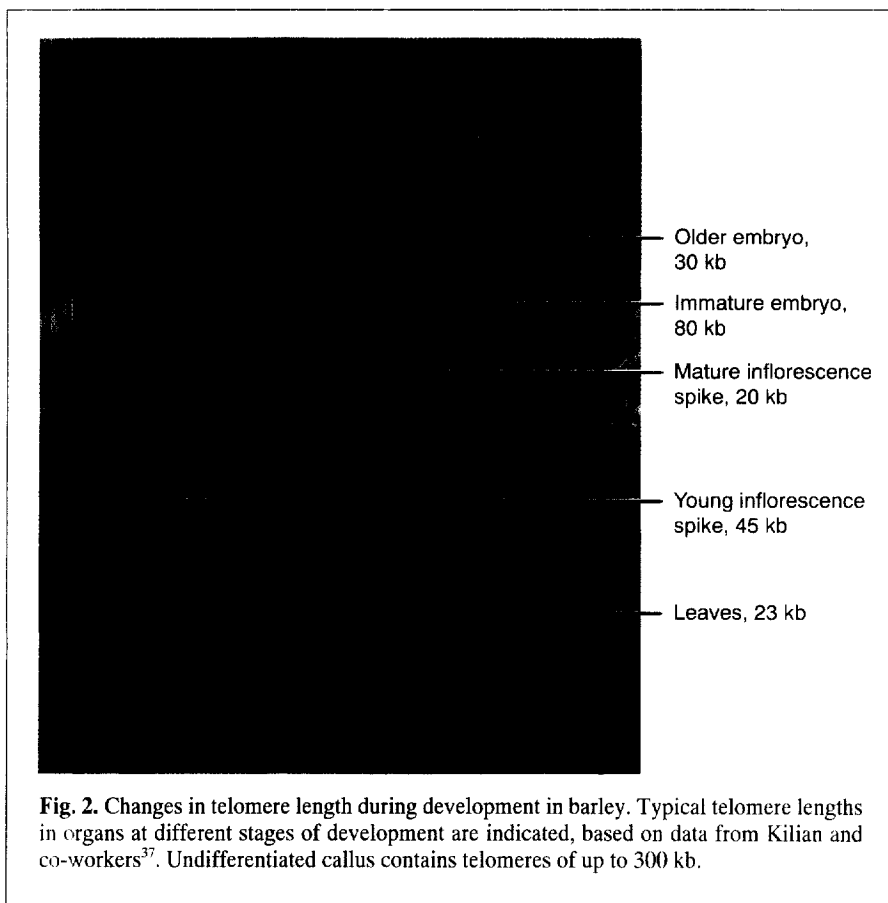spike, 45 kb

— Leaves, 23 kb

Fig. 2. Changes in telomere length during development in barley. Typical telomere lengths in organs at different stages of development are indicated, based on data from Kilian and co-workers[37]. Undifferentiated callus contains telomeres of up to 300 kb.

broken maize chromosomes originally detected by McClintock[1,2]. Chromosome healing has also been documented in mammals. yeast and several other protozoans, including *Plasmodium* and ciliates[31].

Chromosomes harboring terminal deletions are not unusual in some plants. Wheat and barley stocks carrying a variety of terminally deleted chromosomes have been successfully propagated for many generations[32,33]. *In situ* hybridization studies with TTTAGGG probes indicated that the broken chromosomes were capped by telomeres[33,34]. The site of new telomere formation has been examined in a monosomic addition line of wheat carrying a gametocidal chromosome, which causes chromosome breakage[34]. One chromosome that was cleaved inside an rDNA locus was found to have telomeric DNA joined to 18S rRNA at the breakpoint. The two regions were separated by 39 bp of non-cognate sequence, implying that at least one round of the 'breakage–fusion bridge cycle' occurred prior to new telomere synthesis.

Telomere length regulation

The length of the telomeric DNA tract varies tremendously among different organisms, ranging from <50 nucleotides in some ciliated protozoa to >100 kb in rodents. Within a given organism, telomeres are dynamic, and telomere length is controlled by both genetic and developmental factors[35]. For example, plant telomere length varies widely between species, between varieties of one species, and between plants in a recombinant inbred family. *Arabidopsis* telomeres average 2–4 kb (Ref. 5). At the other extreme, tobacco telomeres extend up to 130 kb (Ref. 36).

Telomere length also varies within a single species. A survey of 22 inbred lines of maize showed that the length of the telomere-repeat tract in leaves varied more than 20-fold, from 1.8 kb in the WF9 line to 40 kb in the CM37 line[6]. Analysis of a recombinant

inbred maize family derived from parental lines with different length telomeres showed complex quantitative genetic control of telomere length. Three loci were found to be responsible for most of the variation, but additional loci had minor effects[6].

Telomere lengths can also differ greatly throughout development within a single plant[37]. In the barley cultivar Golden Promise, telomeres in very young embryos are about 80 kb long, but as the embryos mature the telomeres shorten to about 30 kb (Ref. 37). In leaves of the mature plant, telomeres are usually about 23 kb. Telomere length changes again when the inflorescence begins to develop. The average size of telomeres in young inflorescence spikes is around 45 kb, but as the inflorescence ages this size drops to about 20 kb, close to the telomere size in leaves. Telomere length must increase again, probably during gametogenesis or early embryogenesis, to provide the 80 kb length seen in young embryos. In other barley cultivars examined, the general pattern of telomere expansion and contraction remained the same, although the absolute length of telomeres differed slightly. In undifferentiated callus culture cells of barley, the telomeres inflated to 300 kb (Ref. 37). Changes in telomere length during barley development are summarized in Fig. 2.

Because telomeres appear to carry out their basic functions over a wide range of lengths, it is still unclear why there is active length regulation. There is no obvious phenotypic effect of longer or shorter telomeres in the maize lines, or any obvious advantage in altering telomere length throughout development. The decrease in telomere length seen during barley embryogenesis (a loss of 50 kb in a few cell generations) is far too rapid to be caused by problems with replicating the ends of chromosomes in the absence of telomerase. In any case, telomerase appears to be active in grass embryos (Ref. 38; E. Grace *et al.*, unpublished). The rapid decline in telomere length in barley suggests the existence of an active mechanism for quickly adjusting telomere length. Discrete, extrachromosomal fragments carrying telomeric repeats have been isolated from dormant wheat embryos[39], suggesting that telomeres are actively degraded during embryogenesis. This mechanism might be akin to the recombination mechanism previously described for yeast telomeres, in which excessively long telomeres are adjusted to a normal size in a single cell generation[40].

Telomerase regulation

Telomerase expression is tightly regulated in mammals and correlates with senescence and immortalization[3]. In the human germline and early in development, telomere DNA tracts are longest and then progressively decline with each cell division. Telomere erosion has been directly attributed to inactivation of telomerase. Telomerase is normally present in only the permanently regenerating tissues of the human soma (e.g. hematopoietic tissues and epidermis), and in the germline and early stages of embryogenesis. The enzyme is inactive in all other tissues. Hence, telomerase expression is strongly correlated with the capacity for cellular proliferation.

The developmental plasticity of plants could necessitate more flexible regulation of telomerase activity. Most cells in the mammalian body are terminally differentiated, and have permanently withdrawn from the cell-division cycle. In contrast, many plant cells are totipotent and can resume division and differentiation to give rise to an entire new plant. Surprisingly, however, TRAP assays have shown that telomerase regulation is strikingly similar in plants and mammals. Telomerase is expressed in reproductive tissues, but little or no activity is present in vegetative tissues. Telomerase activity has been found in barley embryos and carpels, and to a lesser extent in anthers; however, no activity was detected in barley leaves[38]. Similar results were reported for soybean[41]. Telomerase activity was absent or barely detectable in most soybean vegetative tissues, including leaves, stems and shoot apices; moderate activity was detected in root tips of seedlings and older plants[41]. The lack of telomerase in the shoot tip is somewhat surprising, considering that it is present in root tips and, in animals, telomerase activity in the soma is associated with tissues that have a potential for long-term proliferation. If telomerase activity is completely absent from the shoot meristem, telomeres should become increasingly short in successive new leaves. However, telomere shortening was not seen in leaves of tomato plants[42]. It might be that telomerase activity is present in the shoot apical meristem, but in too few cells to be detected.

Cauliflower forms a massive inflorescence containing many floral buds, each with its own meristem. Telomerase activity is abundant in this meristem-rich tissue[41], but it has not been determined whether the difference in activity between soybean shoot apices and cauliflower floral buds is because of differences between the species or the type or amount of meristematic tissue. Whatever the reasons for this discrepancy, telomerase is very active in cauliflower floral buds, but not cauliflower leaves. These results support the idea that, in plants, telomerase activity is preferentially associated with reproductive tissues.

Immortalization and telomerase

Telomerase activity is absent in most mammalian tissues and in primary cell cultures derived from them. Telomeres shorten progressively in the cultured cells, and eventually most of the cells die off in a brief period called 'crisis'. A few cells can escape from crisis and emerge to form immortalized cell lines. The lack of telomerase in most somatic animal cells could be a defense against cancer, because cells that proliferate rapidly would eventually deplete their telomeres and die. Unfortunately, in more than 90% of primary malignancies, telomerase has been reactivated; in these transformed cells, telomeres become stabilized[3].

In plant cultures, telomerase activity is also correlated with immortality. Most fully differentiated plant cells can give rise to dedifferentiated callus or suspension cultures under the influence of an appropriate hormone regime. However, unlike animal cell cultures, plant cell cultures do not undergo a crisis, and many of the cells are effectively immortalized at the outset. Telomerase has been found to be highly active in all plant cell cultures examined[36,38,41]. However, in contrast to the situation in animal cultures, where telomeres are maintained at a stable length when reactivation occurs, the telomeres of cultured plant cells can continue to expand. For example, telomeres over 300 kb were reported in one barley culture[37]. This observation suggests that reactivation of telomerase in plant cultures is not necessarily accompanied by increased expression of telomere-binding proteins or other factors involved in controlling telomere length.

Conclusions

The cloning of an *Arabidopsis* telomere[5] and the application of the TRAP assay to plant tissues[38,41] has stimulated molecular analysis of plant telomeres and telomerase almost 60 years after McClintock initially discovered that the ends of normal chromosomes have different properties than the newly formed ends of broken chromosomes. So far, most of the work suggests that plant telomeres and telomerases are very similar to those of other eukaryotes. However, because of their plastic patterns of development, plants offer a unique opportunity to investigate the relationship between telomerase expression and cellular proliferation.

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Notice

The following notification was received on 10 March 1998 from Jeff Schell, Jürgen Schmidt and Richard Walden at the Max-Planck-Institut für Züchtungsforschung, Köln, Germany. We will publish further details as this matter is resolved.

We are experiencing difficulty in independently reproducing some data from our recent paper [Ichikawa, T. *et al.* (1997) Identification and role of adenylyl cyclase in auxin signalling in plants, *Nature* 390, 698–701]. This has led us to inform the scientific community that results appearing in figures should be treated with caution. The results affected are figures 1c, 2, 4a and 4b, which suggest a role for cAMP in auxin-triggered cell division in higher plants.

This caution with published data has arisen as a result of the discovery of a case of scientific fraud in our department. It was found that a technical assistant, on her own initiative, was manipulating data. We only became aware of this recently and we are currently unable to assess when this manipulation of results began. At the moment, experiments are under way to investigate this. However, in the meantime, we would like to draw the attention of the scientific community to our concerns. Currently, results under investigation include the auxin independence of growth of protoplasts transfected by the following cDNAs: *axi1* [Hayashi, H. *et al.* (1992) *Science* 258, 1350–1353; Walden, R. *et al.* (1994) *EMBO J.* 13, 4729–4736]; *axi4* [Harling, H. *et al.* (1997) *EMBO J.* 16, 5855–5866]; and *cyi1* [Miklashevichs, E. *et al.* (1997) *Plant J.* 12, 489–498]. Work described in a letter to *Trends in Plant Science* [Miklashevichs, E. *et al.* (1996) *Trends Plant Sci.* 1, 411] is also under investigation. Furthermore, we are investigating results involving the pathways triggered by auxins and LCOs in inducing cell division [Röhrig, H. *et al.* (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 13389–13392]; and the release of a peptide into the supernatant by LCO-treated protoplasts [John, M. *et al.* (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 10178–10182].

We hope very much that our investigations will clarify our doubts and subsequent publication will independently confirm the general validity of these data. In the meantime, however, we would like to draw the attention of the scientific community to this issue and suggest that these data, for the moment, be treated with caution. We apologize for any misunderstanding that might have arisen from this.

Prof. J. Schell, Dr Jürgen Schmidt and Dr Richard Walden