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Functional characterization of poplar and Arabidopsis wood - related hydrolases by reverse genetics and overexpression in Arabidopsis: Effects on hypocotyl elongation.

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ABSTRACT

Trees and their wood present a very valuable and renewable resource (paper industry, raw materials and constructions). Consequently study of wood formation is an important research field. The wood or secondary xylem is formed from the vascular cambium where meristematic activity takes place. During differentiation of wood cells, there is radial expansion and intrusive tip growth phase followed by the secondary wall deposition and programmed cell death. Cell expansion and intrusive tip growth is regulated by the plasticity of the primary cell wall. This wall is composed of pectin, cellulose, hemicelluloses (xyloglucans in particular), and proteins including some wall residing enzymes that are important for regulating wall plasticity. To study the process of wood cell expansion, I focused on a two kinds of hydrolases expected to be involved in the cell wall plasticity/biosynthesis: xyloglucan endotransglycosylases and cellulases. **Xyloglucan endotransglycosylases /hydrolases (XTHs or formerly XETs)** are enzymes capable of mediating the endocleavage of a xyloglucan molecule and the re-ligation of the cut donor to the acceptor that is another xyloglucan molecule or, in some cases, the water. **Cellulases** on the other hand are β,1-4 endoglucanases and could hydrolyze cellulose or hemicellulose molecules. Both these enzymes exist as multigene families and it is a challenge to determine the function of individual members of these families.

PttXET16A is a XTH that has been previously found as expressed in the wood forming tissues of poplar (Bourquin et al, 2002). Single insert, homozygotic lines of Arabidopsis expressing *PttXET16A* ectopically were obtained. I studied effects of the ectopic expression of *PttXET16A* on the elongation of the hypocotyls in the dark and in the light condition, and the effects on cell sizes inboth light conditions. In both light and dark conditions, I observed the inhibition of hypocotyl elongation and the reduction in the final size of cells in epidermis, cortex and endodermis in different *PttXET16A* transgenic lines. This indicates that PttXET16A enzyme reduces cell wall plasticity probably by incorporation of xyloglucan to the wall and by creation of more cross-links between cellulose microfibrils.

Arabidopsis XTHs have been previously identified in the hypocotyls of the plants induced to form a wood (N. Nishikubo et al., unpublished). Single locus T-DNA insertions mutants of these genes were obtained. I studied the elongation of the mutant hypocotyls and cell sizes in the hypocotyl in the light and dark conditions. In six mutants studied: *XTH4, XTH9, XTH16*, *XTH24* and two independent lines of *XTH22*, I observed more or less visible decrease of the hypocotyl elongation only in the light and minor effects on the cell sizes in both experimental conditions. I presume that the XTH genes play role in the hypocotyl elongation in the light causing more cell division but no cell elongation because cell size was not changed in the mutant. To test if XTH genes have redundant functions, double mutants were created and I isolated homozygotic double mutant lines of *XTH9 x XTH22*, *XTH4 x XTH9* and *XTH22 x XTH4*.

PttCEL9B is a cellulase expressed in the meristematic and expanding cells of the wood-forming zone of poplar. Its expression pattern suggests a function in xylem cell expansion. Single insert, homozygotic lines expressing *PttCEL9B* ectopically were studied for effects on the hypocotyl elongation in the light and dark condition. I observed

a big increase of the hypocotyl length in the light but no differences in the dark. These results suggest that *PttCEL9B* activity is important for hypocotyl cell expansions only in light conditions.

KORRIGAN is a cellulase implicated in the cellulose biosynthesis in Arabidopsis. Its weak mutant allele, *irx2*, has a collapsed vessel phenotype, and the poplar orthologue of this gene is up regulated during the secondary cell wall synthesis in developing wood, suggesting a function for KORRIGAN in the wood formation. I studied the hypocotyl elongation in three mutants: *kor1-1*, which is a strong mutation, *irx2-1* and *irx2-2* –less serve mutations. I observed the inhibition in the hypocotyl length in the light condition in *irx2-2* and in the dark condition in *kor1-1*. This result confirms that KORRIGAN endoglucanase can weaken cell wall and its absence could increase wall rigidity. Different result was obtained for *irx2-1* mutant, which had longer hypocotyls in light conditions than wild type plants. It could be explained by fact that studied lines come from different backgrounds.

Present work focused the hypocotyls elongation and the growth of various cell layers in the hypocotyls on the early postembryonic growth. I demonstrated the growth effects or the lack of effects at this stage for the several overexpressing lines and mutants in two hydrolases, XTH and cellulase. The continued work should examine the secondary growth of these lines.

KEYWORDS; cell wall, wood formation, cell wall plasticity, XET, XTH, cellulases, xylogenesis.

CONTENTS

I INTRODUCTINON

Trees, and their wood constitute a very important raw material that is renewable and could be used in paper industry, construction and other applications. Consequently study of wood formation has been an aim for research through several decades. The wood formation is a part of plant vascular differentiation that occurs during the secondary growth. During plant development, primary plant body develops from the meristems found in the embryo, *i.e.* from the root and shoot apex. Tissues arising from these meristems are known as primary tissues, and the part of the plant body composed of these tissues is called the primary plant body. Most plants undergo additional growth that thickens the stem and root; such growth is termed secondary growth. It results from the activity of lateral meristems, one of which, the vascular cambium, produces secondary vascular tissues known as secondary xylem (wood) and secondary phloem.

Plant primary and secondary tissues are composed of cells that are initially formed in the meristems and have thin, plastic walls capable of expansion. These walls are named primary. Properties of the primary cell walls determine the direction and the extension of cell expansion, thus they play an important role in the morphogenesis of wood cells. Following cell expansion, developing wood cells form additional wall layers. These layers of wall are called secondary and by definition are formed after cell expansion is completed. The chemical composition of the secondary wall differs substantially from the primary wall.

1. Primary cell walls and cell expansion

The primary cell wall is a complex and dynamic structure. This structure is reorganized during cell growth and differentiation. Consequently it plays a fundamental role in controlling cell morphology. Chemically, it is composed predominantly of polysaccharides together with a small amount of structural glycoproteins, phenolic esters, and enzymes. Wall components may be ionically or covalently bound to each other. Wall-modifying enzymes and expansins, which are believed to play a role in regulation of cell expansion, are also found in the primary cell wall.

Table 1. Components of the primary cell wall.

Phase	Class	Components
Microfibrilar	Cellulose	β(1,4) Glucan
Matrix	Pectin	Homogalacturonan Rhamnogalacturonan Galactan Arabinan
	Hemicelluloses	Xyloglucan Xylan Glucomannan Arabinoxylan Callose
	Proteins	Enzymes Extensin Arabinogalactan-proteins
	Phenolics	Ferulic acid

One of the most important polysaccharides of the cell wall is cellulose which is composed of linear 1,4 linked ß-D glucoses residues crystallized to form microfibrils. The cellulose microfibrils are coated by hemicelluloses: The predominant hemicellulose in primary walls is xyloglucan. It is a branched polysaccharide which has a backbone composed of 1,4 linked β-D hexosyl residues. Other hemicelluloses found in many primary walls include arabinoxylan, glucomannan, and galactomanan. Pectins form a complex polysaccharide that all contain 1,4 linked α-D galacturonic acid.
All of those polysaccharides are organized together in order to form a giant macromolecular network.

The organization and interaction of wall components are not well understood. It is still discussed how wall organization is modified to allow cells to expand and grow. There are four models of wall organization reviewed by Cosgrove (2000, 2001). The first model is the model of Keegstra et al. (1973) In this model the matrix polymers are covalently linked to one another and cellulose is linked by hydrogen bonds to xyloglucan. Keegstra et al. (1973) proposed that an acidification could lead to the weakening of the H-bonding between xyloglucan and cellulose, and consequently allow the microfibril slippage. The second model is the "tethered network" model of Hayashi (1989). In this model hemicelluloses are important physical organizers of wall architecture. Single chains of xyloglugan make linkages between different microfibrils of cellulose. The "multicoat" model of Talbott and Ray (1992) assumes that non-covalent associations make the linkage between the different layers of polysaccharides. Cellulose is coated with successively looser layers of matrix polysaccharides. The "stratified" wall model of Ha et al. (1997) assumes that strata of pectic polysaccharides separate cellulose-xyloglucans lamellae. Moreover those pectic polysaccharides are thought to control wall extension.

The primary walls contain many enzymes able to modify matrix polysaccharides (Cosgrove, 2001). Endoglycanases cleave the backbone of matrix polysaccharides. Glycosidases remove side chains of polysaccharides. Transglycosidases cut polysaccharides and ligate them together (ex: XTH). Esterases remove methyl groups from pectins and cleave ester linkage between polysaccharide chains. Peroxidases form or break phenolic linkages. All these hydrolytic enzymes may physically weaken the wall but they don't induce wall extension unless there is the turgor pressure in a cell. Turgor pressure can induce wall extension if the cell wall has been weakened by hydrolytic enzymes (McQueen-Mason and Cosgrove 1997).

2. Secondary cell wall.

The secondary cell wall is deposited inside the primary wall after the cell has stopped growing and the primary wall is not longer increasing in surface area. Secondary walls are particularly important in specialized cells that require strong walls to function, for example the xylem cells which are functionally competent in water transport and mechanical support. The protoplast often dies after the secondary wall has been laid down.

Cellulose is more abundant in secondary walls than in primary walls, and pectins are lacking; that's why the secondary cell wall is strong. The secondary walls of cells

found in wood commonly contain lignin. Lignin gives these walls rigidity and hydrophobicity. The matrix of the secondary wall is composed of hemicelluloses. Glycoproteins and enzymes are also present in secondary cell walls.

We can find threes different layers namely S_1, S_2 and S_3, for outer, middle, and inner layer, respectively in the secondary wall, more layers can be found in specialized cells. A layer differs from two adjacent layers in the orientation of its cellulose microfibrils.

3. Aim of the study

The XTH family members encode enzymes capable of mediating the endocleavage of a xyloglucan molecule and the segment transfer to either xyloglucan molecule (transglycosylase activity) or to water (hydrolase's activity) (Hyodo at al. 2003, Iliev et al. 2002, Rose at al. 2002). In *Arabidopsis*, those genes encode 33 different proteins namely XTH1 to XTH33. This family of proteins can be subdivided into 3 distinct subfamilies based on their sequence similarities (Nishitani 1997).

Cellulases are the enzymes hydrolyzing internal 1,4-β-glucosidic bonds such as found in cellulose and hemicelluloses. Most plant cellulases are lacking the cellulose-binding domain and presumably cannot digest crystalline cellulose as microbial cellulases do. Plant enzymes form a multigene family composed of nine subfamilies that differ in enzyme structure and expression pattern. Some members are cell wall residing proteins that have been associated with wall disassembly during abscission or fruit ripening while others were found in rapidly growing cells and are thought to promote wall loosening. The dwarf mutant *korrigan* (*kor 1-1*) has been isolated by T-DNA tagging. KOR is a member of the endo-1,4-β-glucanase family of proteins implicated in the cell wall assembly during cell elongation. The *irregular xylem 2* (*irx2*) mutant of *Arabidopsis thaliana* is cellulose deficient in the secondary cell wall (Szyjanowicz et al, 2004). This mutant is formed by a point mutation in the *KOR* gene.

Several cellulases and XTHs were identified in developing wood in poplar (Mellerowicz et al 2001). Among them *PttXET16A* was proposed to function in secondary wall developing fibers (Bourquin et al 2002). In addition, several members of XTH family have been found in secondary xylem forming *Arabidopsis* hypocotyls (Nobuyuki Nishikubo et al, in preparation). Mutants in these genes were obtained from Syngenta and purified to single insertion locus, homozygotic lines. Gene encoding a cellulase, *PttCel9B*, was fund expressed in expanding xylem cells and cambium (Junko Takahashi et al in preparation). And finally KOR gene was shown to affect secondary walls of xylem (Szyjanowicz et al, 2003). Mutants of this gene *kor 1-1* as well as *irx2-1* and *irx2-2* respectively coming from L*er* and Col backgrounds are functionally studied in the present study.

The aim of the present study was to determine the putative involvement of selected members of XTH and cellulase families in wood formation. The functional characterization is carried out either by overexpression or by mutant analysis in *Arabidopsis*.

II MATERIALS AND METHODS

1. Arabidopsis lines used in study.

Ecotypes

Ecotypes: Landsberg *erecta* (L*er*), Columbia (Col), and Wassilewskija (Ws) were used in a pilot experiment. These ecotypes served as wild-type controls for the experimental transgenic or overexpressing lines.

Overexpressing lines

Overexpressing lines were obtained in Col background. They carried a construct containing a coding sequence of a cellulase (PttCel9B, GenBank accession number not registered.) or a XET (*PttXET16A*, GenBank accession number AF515607) fused behind the 35S promoter. The lines were homozygotic and contained single inserts.

Mutants

XTH single mutants

Knockout *Arabidopsis* lines were obtained from SYNGENTA and had a T-DNA insertion in one of the XTH genes (Table 2). The original lines were purified to single locus insertion in a gene of interest by a series of back-crossing prior to this study, and homozygotic lines were used in this study.

Table 2. List of XTH genes studied

Gene	Other name	AGI number
XTH4	EXGT-A1	At2g06850
XTH9	XTR16	At4g03210
XTH16		At3g23730
XTH22	TCH4	At5g57560
XTH24	MERI5	At4g30270

Purification of XTH double mutant lines

The single mutant lines were cross-pollinated according to Table 3 and the progeny was screened using PCR with primers specific for each XTH gene and the left border of T-DNA.

Table 3. Double XTH mutants purified

XTH9 x XTH22
XTH4 x XTH22
XTH4 x XTH9

DNA was extracted from a leaf of each plant according to Edwards et al. (1991). PCRs were performed in 20 μl volume with following concentrations: 1 X Taq polymerase buffer, 200 μM d NTP mix, 0.5μM of each primer, 5μl of template DNA
Temperature cycling:

- 94^{o}C for 30 sec (denature)
- 56 ^{o}C for 40 sec (anneal)
- 72^{o}C for 50 sec (elongate) (60 sec per kb target sequence length)
- 34 cycles
- 72^{o}C for 5 min at end to allow complete elongation of all product DNA

Cellulases

The following *KOR* mutants were used: *kor1-1*, *irx2-1* and *irx2-2*.

2. Growth conditions on the plates.

Seed sterilization

Seed of *Arabidopsis* WT and mutants were surface sterilized in 70 % ethanol with a drop of Tween 20 (one drop in 50 ml of 70% ethanol) and vortexed every 2 minutes during 10 minutes. This solution was replaced by 95 % ethanol, in two changes, with vortexing in between. Finally the ethanol was removed with a pipette and all seeds were left to dry over night in a sterile hood.

Growth on plates

Seeds were plated on a nutrient-solidified medium 0.5% (w/v) Murashige and Skoog (MS) 0,5 % (w/v) sucrose and 1,5% (w/v) agar, pH 5.7. Seeds were placed on the agar surface with a sterile spatula and plates were sealed with parafilm and stored in a refrigerator for vernalization treatment for 3 days. Subsequently plates were placed vertically either in the light conditions (16 hr light, 8 hrs dark) or in the dark conditions (wrapped in aluminum foil), in the controlled temperature chamber (temp 23°C). Dark germinating seedlings were exposed to light in the sterile hood for 4 hours before dark growth, in order to break skotodormancy.

3. Measurements of hypocotyl length.

Pictures of growing seedlings were taken at different stages of growth (days 1-6) with a stereomicroscope equipped with a camera. Hypocotyl length was measured with Image J 1.31v (http://rsb.info.nih.gov/ij/java1.3.1_03). Each point represented a mean of ten measurements.

4. Measurements of cells in the young hypocotyls.

Seedlings were cleared and stained in Congo red using Malamy and Benfey (1997) protocol for clearing *Arabidopsis* roots (Appendix). Pictures of the hypocotyls were taken using the AxioVision camera on a Zeiss Axioplan2 microscope and the cells were measured using the AxioVision software. For each data point 5 cells of each layer of 5 seedlings were measured.

5. Statistical analysis.

Data were analyzed by the analysis of variance using a GLM procedure of SAS program (Release 6.03). Least-square means were computed for all effects and meaningful comparisons between pairs of corrected means were carried out with the LSMEANS SAS option. Differences between means with probability values ≤ 0.05 were considered significant.

III RESULTATS AND DISCUSSION

1. Hypocotyl elongation and cell expansion in the wild type plants

The ecotypes used, Ws-Wassilewskija L*er*-Landsberg *erecta* and Col-Columbia, are the background lines for different mutants and overexpressing lines studied later. Some parameters of hypocotyls elongation were therefore established for these ecotypes to provide some background information. Hypocotyl elongation was studied in the light and dark condition.

a. Time course of hypocotyl elongation

Seeds were placed in three parallel lines on vertical plates, Col was placed on the top, L*er* in the middle and Ws were placed on the bottom of the plate. Seeds were vernalized and left in the growth chambers for a period up to 7 days in. Each day starting from day 2, a plate with all three ecotypes was taken for measurements.

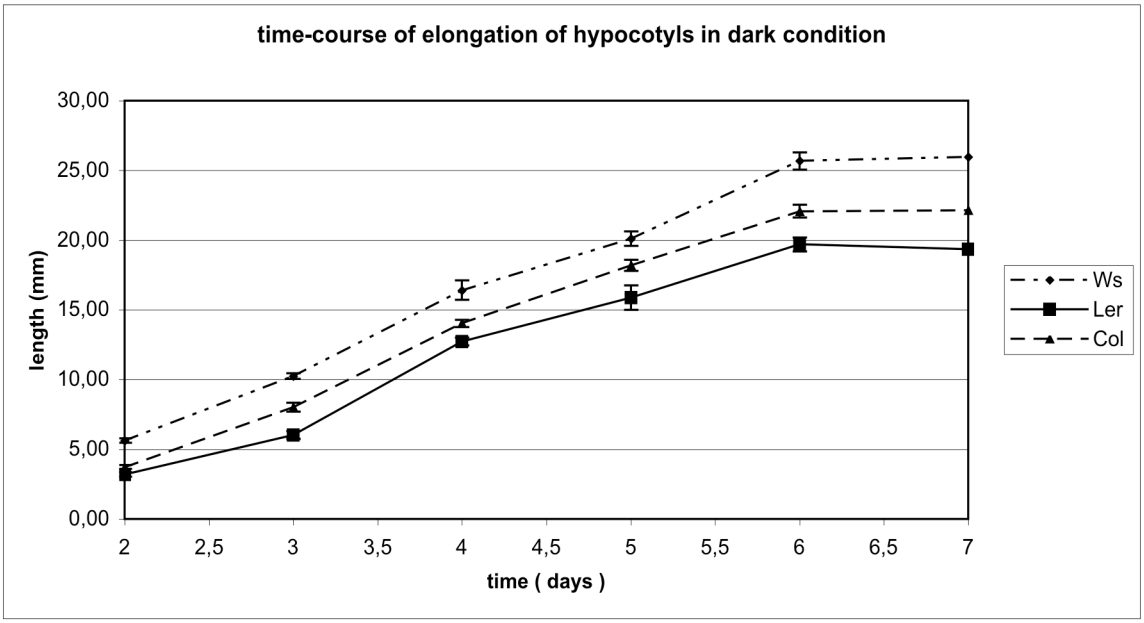


Fig 1. Hypocotyl length in the dark condition. Errors bars represent SE. Values are the mean of 10 measurement / day/ecotypes. Ws: Wassilewskija; L*er*: Landsberg *erecta;* Col: Columbia

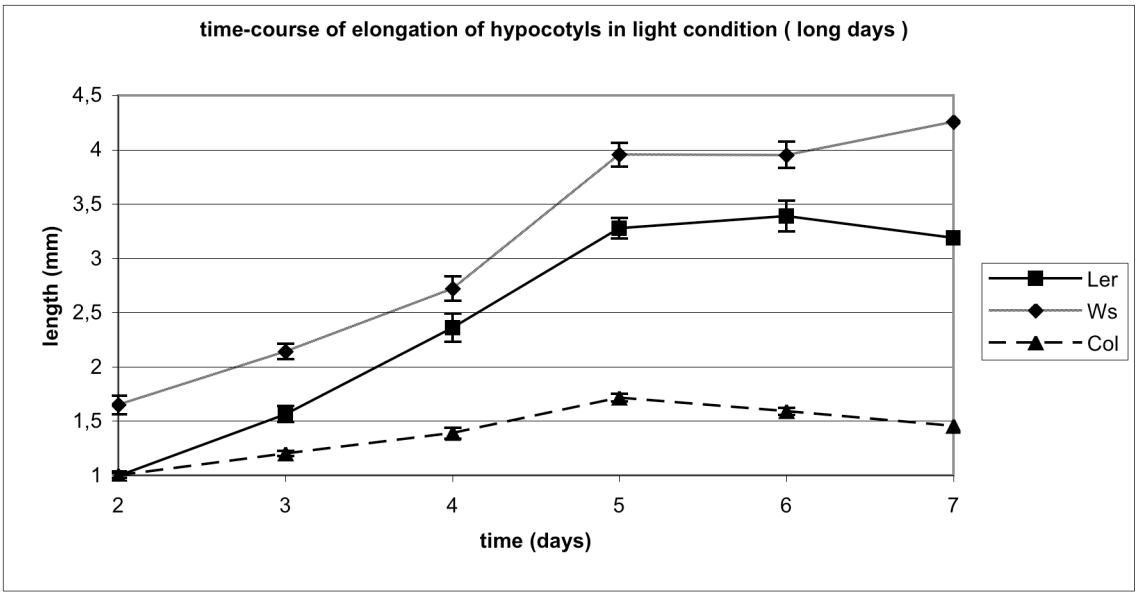


Fig 2. Hypocotyls length in the light condition. Errors bars represent SE. Values are the mean of 10 measurement / day/ecotypes. Ws: Wassilewskija; L*er*: Landsberg *erecta;* Col: Columbia

In the dark condition (Fig 1), the growth continued at a relatively stable rate from day 2 until day 6 when the elongation seemed to stop in all ecotypes. Transferring seedlings to the light after 6 or 7 days in the dark did not stimulate any further growth (data not shown). Growth curve was similar for three ecotypes. There was only a small difference in the initial hypocotyl length that persisted throughout the elongation period. The longest hypocotyls had Ws ecotype and the shortest L*er* ecotype. The seedlings grew about 5 mm per day during the period.

In light condition, the elongation of the hypocotyls seems to stop after 5 days (Fig 2). There was about a 3-fold difference between the longest (Ws) and the shortest (Col) hypocotyls. L*er* seedlings had an intermediate behavior. Ws and L*er* seedlings grew about 0.5-1.0 mm per day while Col seedlings grew more slowly about 0.25 mm per day and had shorter hypocotyls than other ecotypes.

b. Cell elongation in four different tissues layers of hypocotyl

Growth in general can result from cell division and/or cell expansion. I were interested if the hypocotyl elongation can be explained by the cell elongation as is frequently assumed (Gendreau et al 1999) and if this assumption could apply to different cell layers in the hypocotyls: epidermis, first and second cortex layer, and endodermis. Fig. 3 presents a diagram of different cell layers measured in hypocotyls.

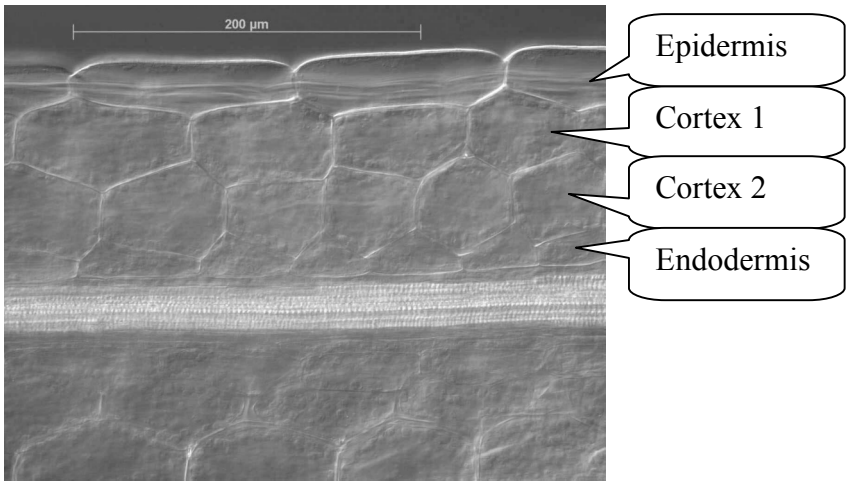


Fig 3. Col hypocotyl at day 3 in the light condition stained with Congo red.

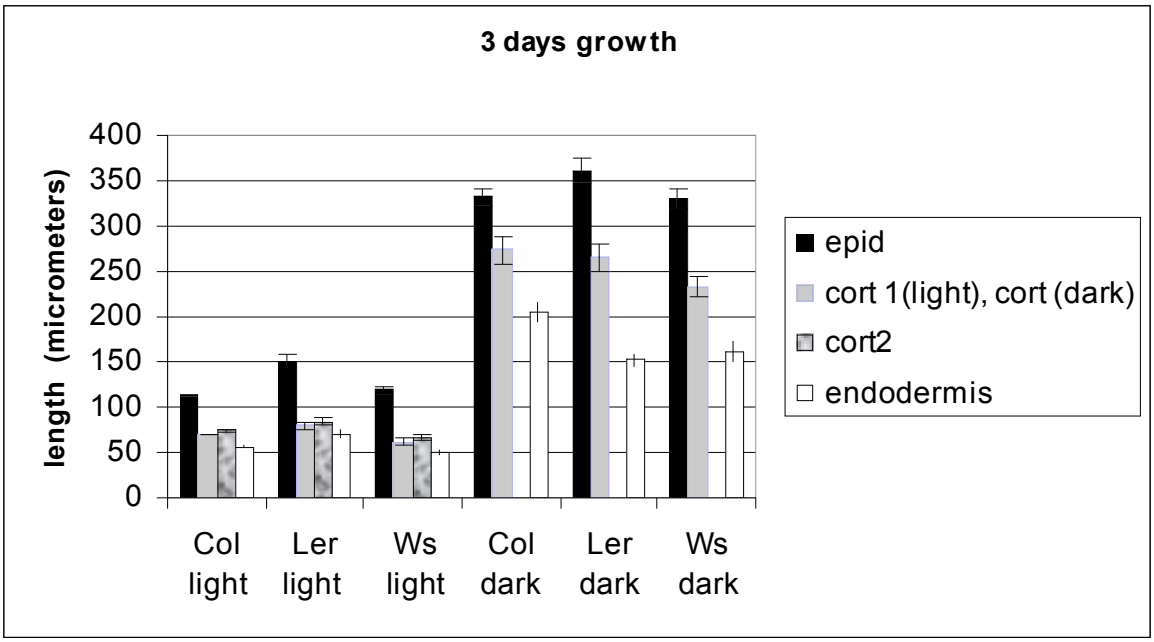


Fig 4. Cell length in the middle of hypocotyls in the light and dark condition. Error bars represent SE. Values are the means of 25 measurements. L*er*: Landsberg *erecta,* Col: Columbia, Ws: Wassilewskija, Epid: epidermis, Cort: cortex (first and second layer).

Hypocotyls of all three ecotypes had three cortical cell layers below the epidermis, named here cortex 1, cortex 2, and an endodermis (Fig. 3). For the light-grown seedlings, it was possible to distinguish among these layers based on the anatomical features. For the dark-grown seedling however, the distinction between cortex 1 and cortex 2 was not easy and therefore cells of these two layers were bulked as 'cortex'. All cells measured were located in the middle part of the hypocotyls according to the Fig. 5. In the light grown seedlings, cells of cortex 1 and cortex 2 had similar

length (Fig. 4). In both conditions, epidermis cells were the most elongated, followed by cortex (1 and 2) and the endodermis. Dark grown seedlings had about 3 times longer cells in all cell layers compared to the light grown seedlings (Fig. 4, Table 4). Expected number of cells in the hypocotyls were different for three ecotypes and light or dark conditions. This results needs to be verified.

Table 4. Expected cell number based on the epidermis cell length and hypocotyl length measurements. Measurements in the middle of the hypocotyl after 3 days of growth in the light and dark.

	3 days light			3 days dark		
	Epidermis cell length (μm)	Hypocotyl length (μm)	Expected cell number	Epidermis cell length (μm)	Hypocotyl length (μm)	Expected cell number
Col	112.54	1202	11	333.02	8018	24
Ler	150.87	1560	10	361.02	6030	17
Ws	118.32	2130	18	331.24	10240	28

The hypocotyl length ranking was Ws>L*er*>Col in the light and Ws>Col>L*er* in the dark. In the experimental condition I could detect any relationship between the length of the hypocotyls and the cell size. This could be explained by 2 hypotheses. The first is that the position of the seedlings in the plates mattered, the seedlings placed on the bottom of the plate could have received less light and consequently the rate of growth could increase. Then I would expect Col<L*er*<Ws, as it was observed in the light. Another explanation could be that when I measured the cell size at 3 days, I did not measure fully elongated cells, and the growth gradient is not the same in the different ecotypes.

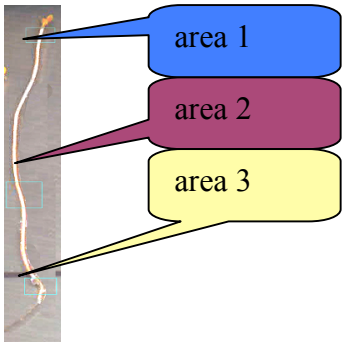
In order to determine the gradient of growth along the hypocotyls in the dark and the light, I measured cell length and diameter in three different areas: area 1 - apical region, area 2 - middle and area 3 - basal region of hypocotyl (Fig. 5A). This will help to determine if there is a big variation of growth along the hypocotyls and if there is a variation of the rate of elongation between the different layers. I used Col seedlings in the mid-phase of hypocotyls elongation i.e. germinated for 3 days in the light or dark.

In the light, the cell size increased from the apical area to the basal area, this gradient was consistent for all cells types (Fig. 5B). There was no big variation of the diameter in all cell files, but we can observe a bigger diameter in the area 2 and this result is consistent in all cell files.

In the dark, the largest cells were found in the middle of the hypocotyls and the smaller ones were in the apical region (Fig. 5C) I did not observe any diameter variation.

The magnitude of growth along the hypocotyls seems to be different in the four different layers (Table 5). If the cells grow symplastically, i.e. together without any sliding of one layer past another one, then the different rate of growth reflects the disproportional occurrence of cell divisions among cell layers or perhaps the asynchronous transition from meristematic to expanding phases of growth in different cell layers. Cells that divided more recently would have a smaller growth increment. In

the light, the epidermis cells increased proportionally less than other cell layers. Cortex 1 increased the most. It appears that most recent divisions would have occurred in the epidermis, and some endodermis/cortex 2 cells divided more recently than cortex 1. Indeed, I have observed cell division in the epidermis in the light condition related to the formation of stomata. In the dark, most recent cell division seemed to occur in the cortex



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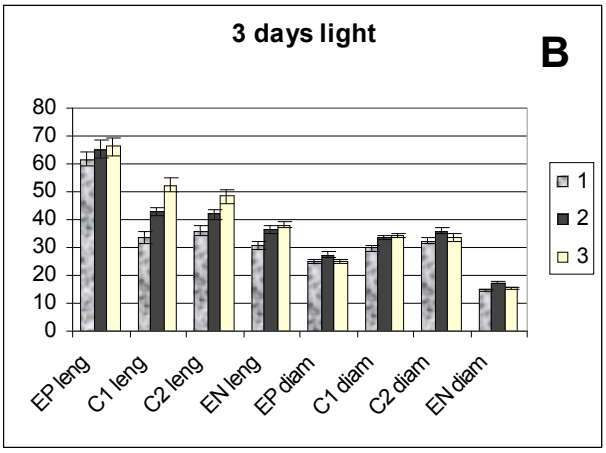


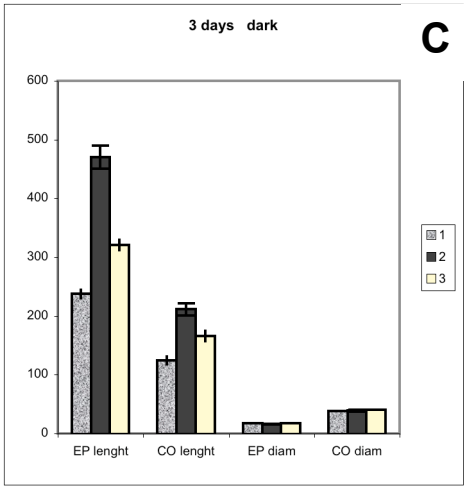


Fig 5. Variations in cell dimentions in different cell layers along the length of hypocotyl. **(A)** The position of the measurements along the hypocotyls. **(B)** Cell length and diameter in areas 1-3 in the light. **(C)** Cell length and diameter in areas 1-3 in the dark. Means of 25 measurements/layer. EP – epidermis,C1, - cortex 1 C2,- cortex 2, CO – cortex EN - endodermis. Errors bars represent SE

Table 5. Differences in cell sizes in different cell layers between different areas along the hypocotyls (as shown on Fig 5A).

	Light		Dark	
Layer	Area 1 to 2 (increase in %)	Area 1 to 3 (increase in %)	Area 1 to 2 (increase in %)	Area 1 to 3 (increase in %)
Epidermis	5%	7.3%	98%	35,26%
Cortex 1	27%	55%	69,4%	33,1%
Cortex 2	16%	34%		
Endodermis	18%	23%	-	-

c. Hypocotyl elongation and cell expansion of the wild type plants: discussion

The aim of these experiments was to learn about the growth dynamics and distribution in the light and dark in *Arabidopsis* hypocotyls of three ecotypes used later as controls.

Ecotypes differed in hypocotyl elongation. Most striking was the difference between Ws and Col in the light, with Col having three times shorter hypocotyls. However I cannot exclude some influence of seedling position confounding the effect of ecotype in the light and these measurements have to be repeated with a different seedling placement on the plate.

We found that hypocotyl completes its elongation within 6 days in the dark and 5 days in the light in the conditions used. Dark grown hypocotyls could not further elongate when transferred to light after 6 days of growth. The rate of growth was between 2 to 4 times faster in the dark compared to light so hypocotyls growing in the dark were significant longer.

There are two major factors controlling the cell size and cell expansion. The first one is the action of cell wall enzymes acting on wall plasticity, such as XTH, cellulase, expansin implicated in cell wall biosynthesis and modification (Gendreau et al, 1999), and the second is the turgor pressure. These direct growth agents are in turn controlled by other factors. One of them is the level of ploidy, which is known to influence cell volume and consequently cell elongation (Kondorosi et al. 2000). A strong correlation between cell size and endoreduplication has been reported for a range of species (Melaragno et al., 1993). A significant DNA replication activity during hypocotyls development was observed (Gendreau et al., 1999). This replication was related to endoreduplication cycles.

The other important factor of hypocotyl elongation is the effect of hormones. Gibberellins (GAs) are implicated in hypocotyl elongation and required for dark-germinated seedlings (Gendreau et al., 1999). GA and ethylene promote hypocotyl growth via cell elongation. Auxin increases the production of ethylene and mediate cell elongation. Recently Rashotte et al. (2003) have demonstrated the existence of a basipetal transport of two natural auxins, indole-3-butyric acid and indole-3-acetic acid in Arabidopsis hypocotyl. These two hormones had a different effect on hypocotyl elongation: IBA but not IAA was able to stimulate hypocotyl elongation in high light condition, at concentration ranging from 1 to 10 uM. IAA inhibited hypocotyl elongation in high light, low light and dark condition. It is possible that the auxin could inhibit cell growth in the apical area because of its high concentration and in the basal area it could be a limiting factor in the light condition and consequently inhibit cell elongation. In the dark the auxin transport is not required for hypocotyl elongation and consequently a difference of cell size along the hypocotyl could not be explained by the transport of the auxins.

2. Effects of *PttXET16A* overexpression on the hypocotyl elongation and cell expansion.

a. *PttXET16A* overexpressing lines

Six single insert *PttXET16A* overexpressing lines that were generated using a vector with 35S promoter (N. Nishikubo et al in preparation) were analyzed for the effects on hypocotyl elongation in the light and dark conditions, and effects on cell sizes. The expression levels for the lines were determined previously (Fig. 6). Line 1 was expressing approx 2-3 times less than other lines.

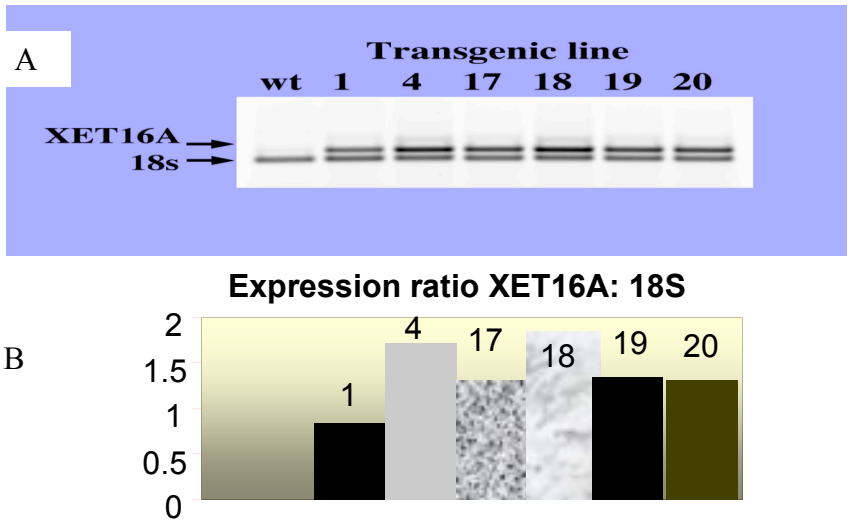


Figure 6. Expression levels of *PttXET16A* in the transgenic lines used in this study. A- Et-Br- stained gel showing the result of quantitative RT-PCR. 18S rRNA was amplified as a standard. B – corresponding densiometry figure. Data from N. Nobuyushi – in preparation.

b. Time course of hypocotyl elongation in *PttXET16A* overexpressing lines

In the light, three different groups can be distinguished (Fig. 7):
wild type and lines 1 and 18, had the longest hypocotyls, ii) lines 17, 19 and 20, which had distinctly shorter hypocotyls, and iii) line 4 with the shortest hypocotyls. In all lines hypocotyls grew between 0.17 and 0.20 mm per day and reached a plateau between day 5 and 6. For all lines exception line 18, I can interpret these results in relation to the *PttXET16A* expression level: the stronger expression, the shorter hypocotyls. Thus, it

appears that overexpression of *PttXET16A* typically results in the inhibition of hypocotyl elongation.

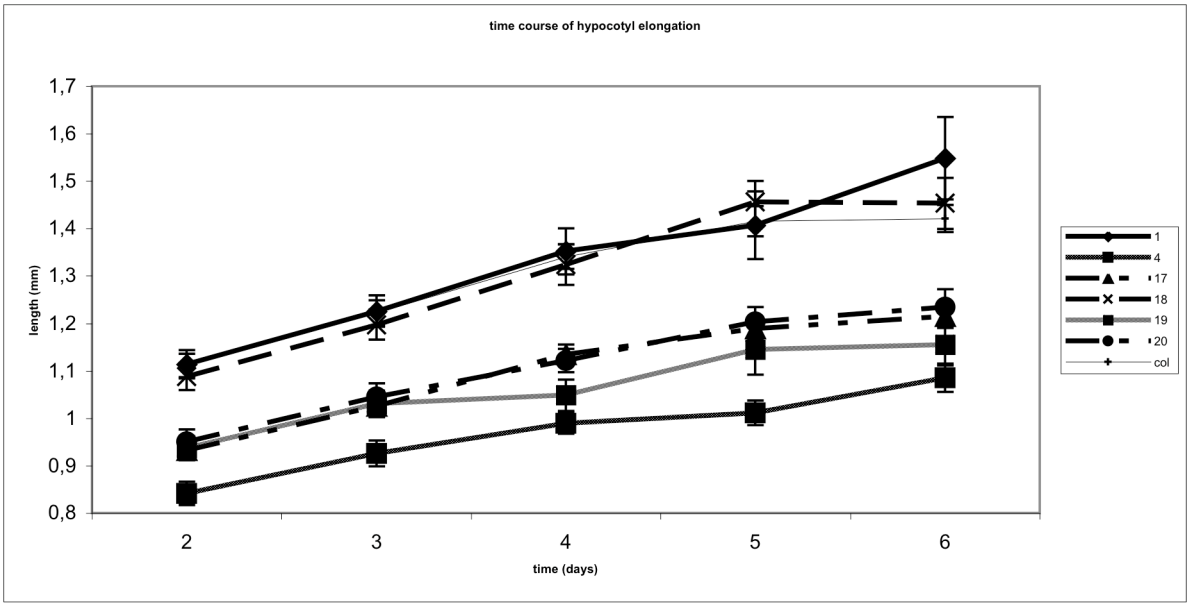


Figure 7. Hypocotyls length in light condition of XET16A lines: 1, 4, 17, 18, 19, 20 and Col. Errors bars represent SE. Values are the mean of 10 measurement / day/genotype.

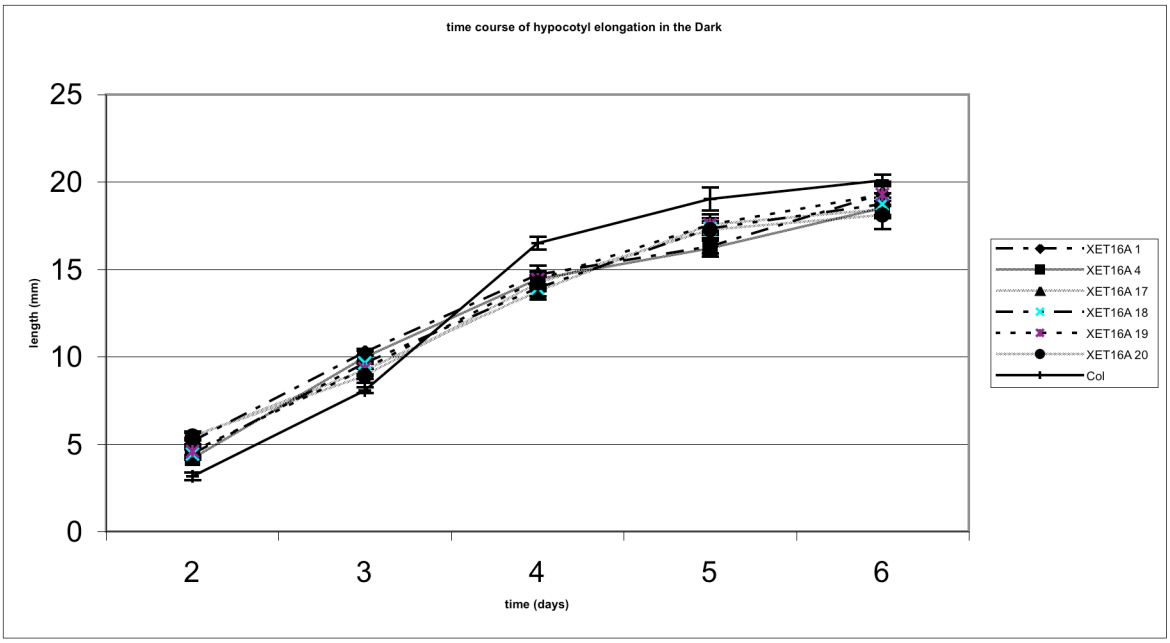


Figure 8. Hypocotyls length in dark condition of XET16A lines 1, 4, 17, 18, 19, 20 and Col Errors bars represent SE. Values are the mean of 10 measurement / day/genotype.

In the dark all the transgenic lines exhibited the same behavior: they grew faster at the beginning (until day 3) and slower later (days 4 to 6) (Fig. 8).

c. Cell elongation in hypocotyls of *PttXET16A* overexpressing lines

To investigate if the differences in the hypocotyl length corresponded to the differences in cell sizes, I measured cell lengths in the epidermal, cortical and endodermis cell layers for each genotype at day 3 and 6.

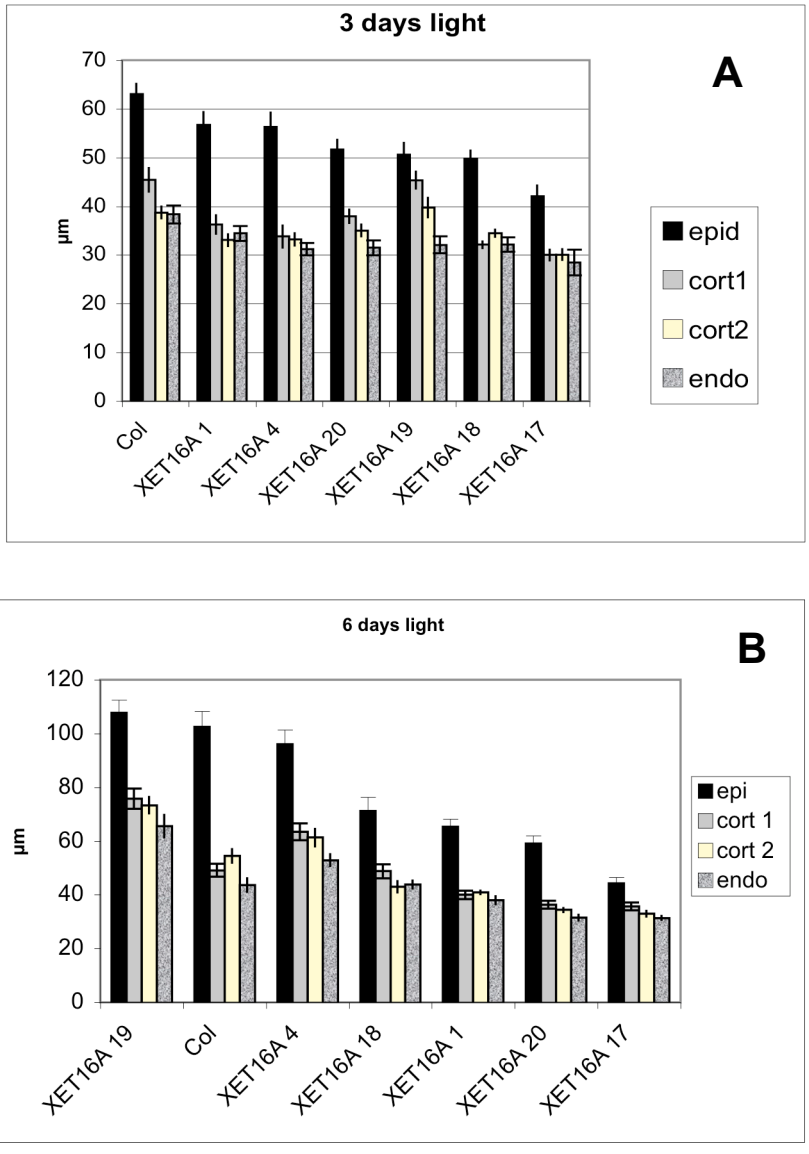


Figure 9. Cell length, in the middle of the hypocotyls, in light condition at day 3 (A), and at day 6 (B) of XET16A lines 1, 4, 17, 18, 19, 20 and Col. Errors bars represent SE. Values are means of 25 measurement / day/genotype.

In the light condition after 3 days, all lines overexpressing *PttXET16A* had shorter cells of the epidermis and endodermis (Fig. 9A). The cortex cells (cortex 1 and cortex 2) were shorter in the all lines except line 19, where they were longer. Thus overall, there was a reduction in cell length, in all cell layers in majority of overexpressing lines. Inconsistency for line 19 cortex might be due to the fact that the cells were not fully elongated and were at the different phases of elongation in different lines. After six days, this variability should not play a role since the hypocotyls were fully elongated. At this time, epidermis cells were visibly shorter in lines 1, 17, 18, and 20, but not in lines 4 and 19 (Fig. 9B). Cortical and endodermis calls were shorter in lines 1, 17 and 20, but they were longer in lines 19 and 4.

In the dark condition, at day 3, the hypocotyls of *PttXET16A* overexpressing lines were longer than the wild type (Fig. 8). But only line 4 had significantly longer cells in both epidermis and cortex, and line 19 had longer cortical cells (Fig. 10A). Cortical cells of lines 1, 18 and 20 were shorter than wild type. In fully elongated hypocotyls of transgenic lines at day 6, which were shorter than the wild type, the epidermis and cortex cells were also shorter, but endodermis cells were longer (Fig. 10B). This unpredictable behavior suggests differential effects of *PttXET16A* on cell division and elongation in different lines

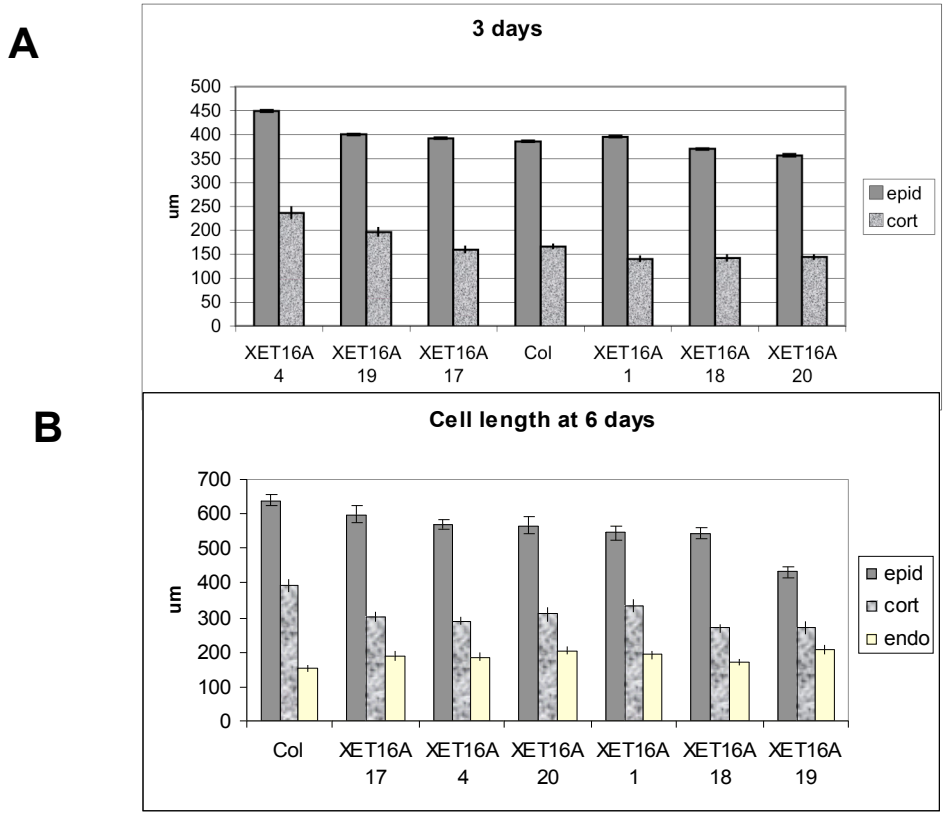


Figure 10. Cell length, in the middle of the hypocotyls, in dark condition at 3 days (A), and at 6 days (B) of XET16A lines 1, 4, 17, 18, 19, 20 and Col. Errors bars represent SE. Values are the mean of 25 measurement / day/genotype.

d. Effects of *PttXET16A* overexpression on the hypocotyl elongation and cell expansion: discussion

Generally *PttXET16A* overexpression resulted in inhibition hypocotyl elongation. In the light, in all lines except 18 there was a visible dependence: the stronger expression level, the shorter hypocotyls. In the dark, all lines showed the same overall inhibition of the final hypocotyls length, but during the early stage of growth (days1-3), all the lines had longer hypocotyls than WT.

Considering only fully elongated hypocotyls, there seems to be a limited correlation between the hypocotyl length and cell length. For example, the correlation was observed in the dark for epidermis and cortex but not for endodermis. In the light, lines 4 and 19 had shortest hypocotyls but longest cells. These results could be explained by an inhibition of hypocotyl elongation by PttXET16A by two different mechanisms, inhibiting cell expansion and inhibiting cell division. PttXET16A probably reduces cell wall plasticity by incorporation of xyloglucan to the wall and thus creating a possibility for more xyloglucan cross-links between cellulose microfibrils. Reduced cell wall plasticity might also lead to a reduction in the cell division frequency.

3. Effects of knock out mutations in the selected XTH genes

a. Description of mutant lines

XTH genes expressed in the Arabidopsis wood-forming hypocotyl were identified and the knock out lines carrying T-DNA insertions were obtained from TAIR stock center (http://www.arabidopsis.org/index.jsp). Six lines were backcrossed until a single insert corresponding to the target XTH gene was obtained and homozygotic seed lines were used in this study. The knock-out was complete as judged by the absence of XTH mRNA in the quantitative RT –PCR in the XTH4 and XTH22B line, and partial in XTH9, XTHJ22A and XTH24 lines (Nobuyushi Nishikubo et al. in preparation). Line XTH16 did not show any reduction in transcript level as compared to the WT (Fig. 11).

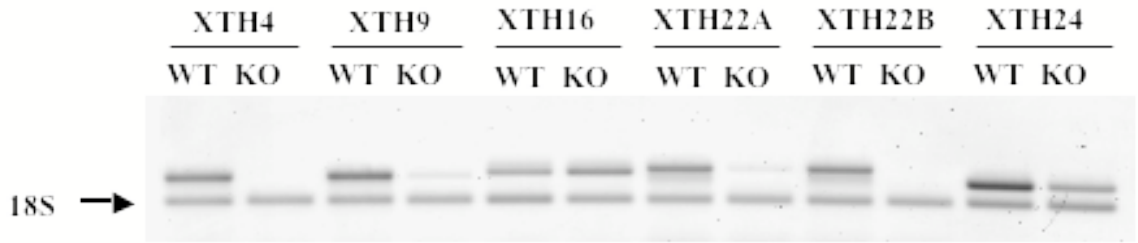


Figure 11. Quantitative RT-PCR showing the level of XTH expression in the knock-out (KO) lines compared to the level observed in the WT in 10 day-old seedlings. Data from Nobuyushi Nishikubo.

b. Time course of hypocotyl elongation in the light condition

In all the lines, the hypocotyls elongation was inhibited (Figure 12). XTH9 line behaved somewhat erratically showing a large hypocotyl length increase between day 4 and 5. Most length reduction was observed in lines XTH24 and XTH22A. These were partial knock-outs mutations. Surprisingly, the line 22B, complete knock-out in XTH22, did not show such a strong inhibition as the partial knock out XTH22A. Line XTH16 was also reduced in the hypocotyl elongation even though no effect on the gene transcription level was observed in 10 days- old seedlings (Fig. 11).

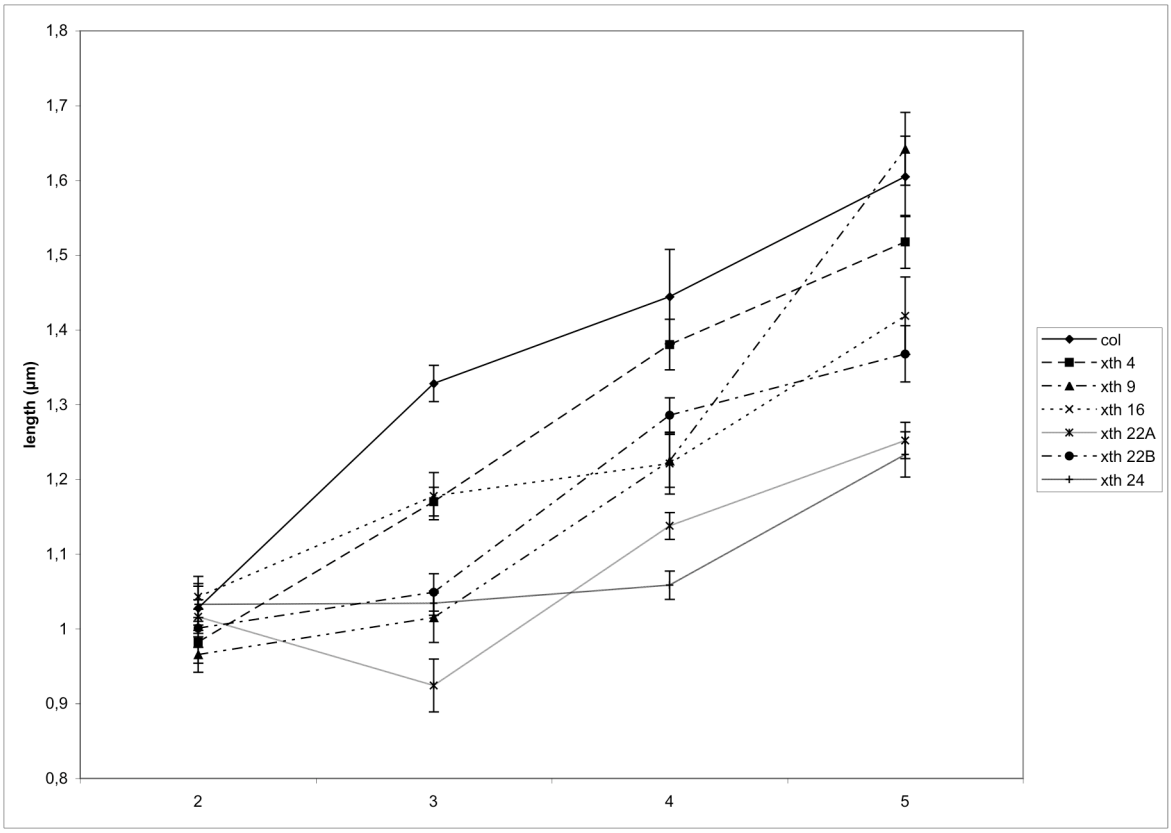


Figure 12. Time course of hypocotyls elongation in the knock-out lines. Errors bars represent SE. Values are the mean of 10 measurement / day/genotype.

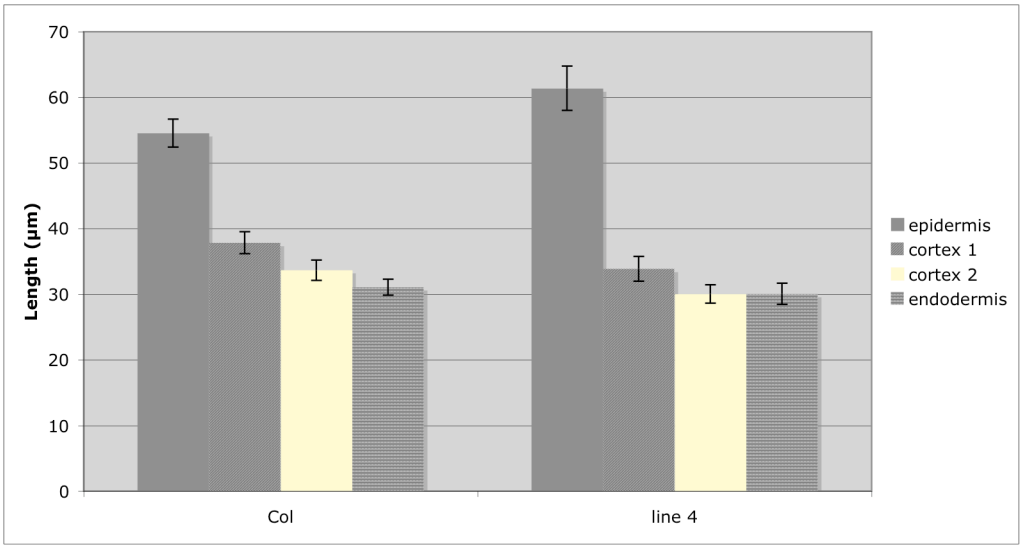
c. Effects of knock out mutations on the cell sizes in different cell layers of light-grown seedlings

In the line XTH4, epidermis cells were longer at day 3 and shorter at day 6 in comparison to WT plant. Cortical cells were shorter at day 3 and similar to WT at day 6 and endodermis cells were shorter at day 6 (Fig. 13). XTH4 hypocotyls were shorter than the wild type. At day 3, the hypocotyls cells were certainly not fully elongated. Moreover, I measured cells localized in the middle of the hypocotyl, and according to the

idea of an gradient of growth, the middle of the hypocotyl at day 3 does not correspond to the same area at day 6. If I focus on day 6 data, hypocotyl length could be possibly explained by reduction in epidermis and endodermis cell length but cortical cell layers must have been affected in the cell division.

XTH4

A



B

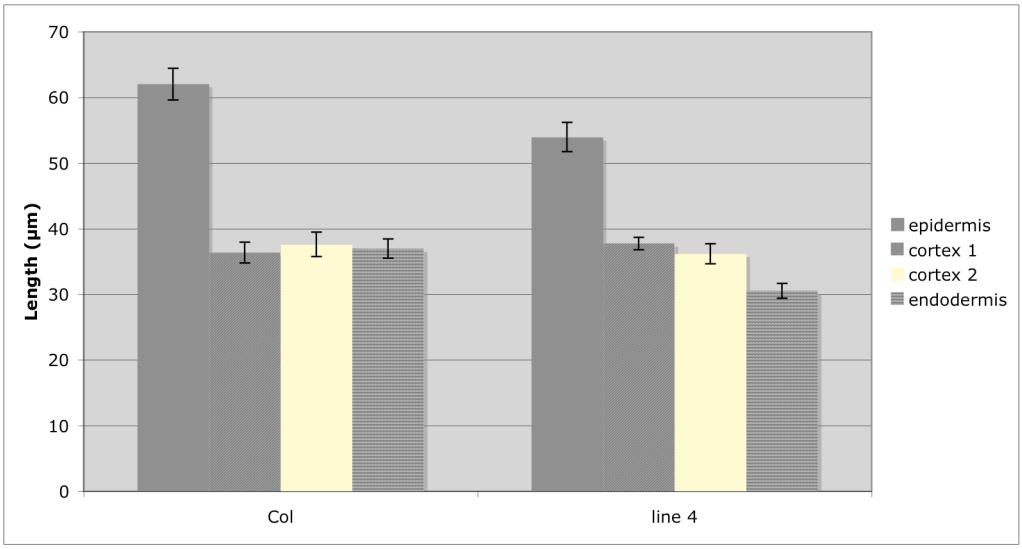
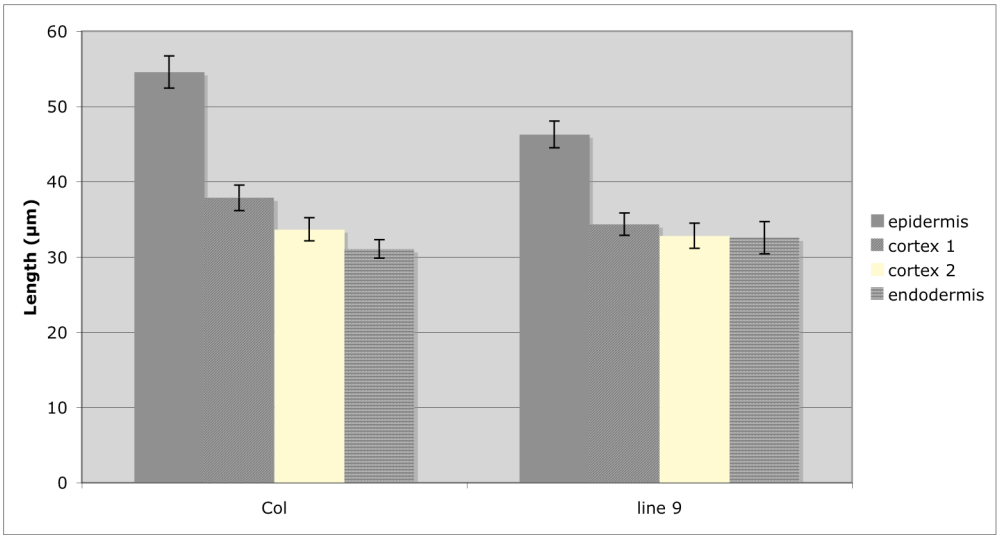


Figure 13. Cell length in the middle of the hypocotyls in light condition at day 3 (A), and at day 6 (B) in knockout line XTH4 and WT-Col. Error bars represent SE. Values are the means of 25 measurement / day/genotype

XTH9

A



B

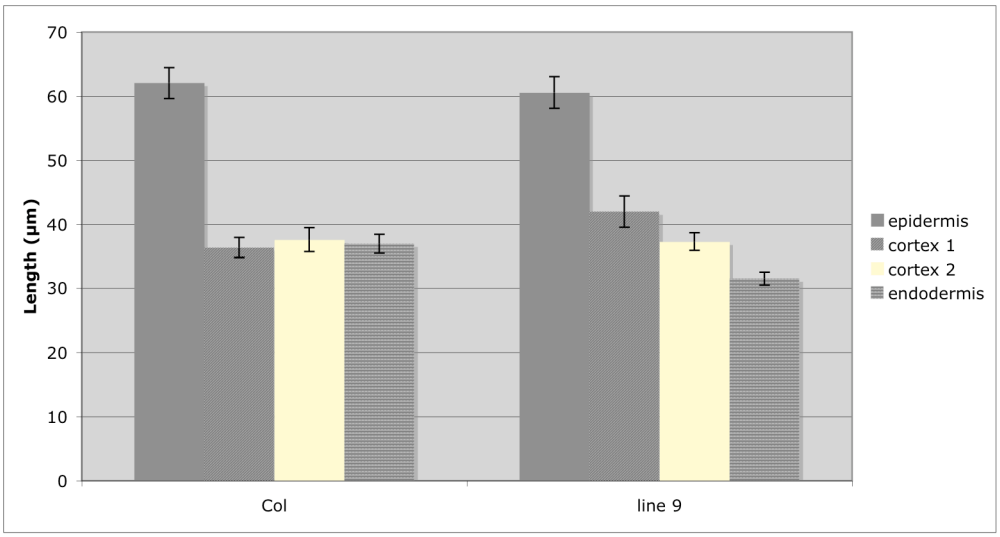


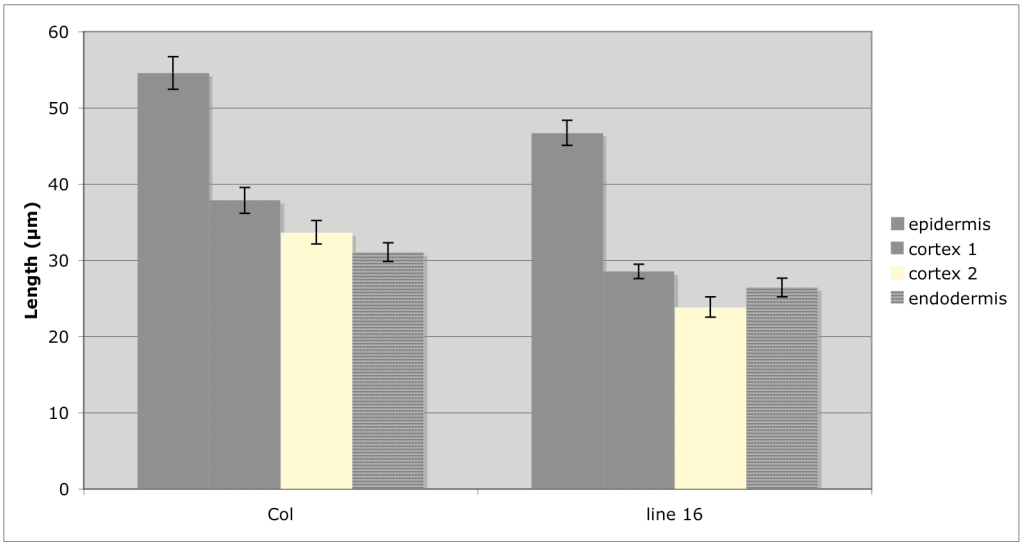
Figure 14. Cell length in the middle of the hypocotyls in light condition at day 3 (A), and at day 6 (B) in knockout line XTH9 and WT-Col. Error bars represent SE. Values are the means of 25 measurement / day/genotype

In line XTH9, epidermis cells were shorter than WT at day 3 and not significantly different at day 6 (Fig. 14). Endodermis cell size was inhibited at day 6. In addition the hypocotyl length of XTH9 line was reduced at day 3 and not affected at day 6. In this case changes in epidermis length at days 3 and 6 correlated with changes in hypocotyl length (Fig. 12, 14). But increase in endodermis cell length would suggest the

upregulation of cell division in this cell layer. This result should be confirmed by cell counting.

XTH16

A



B

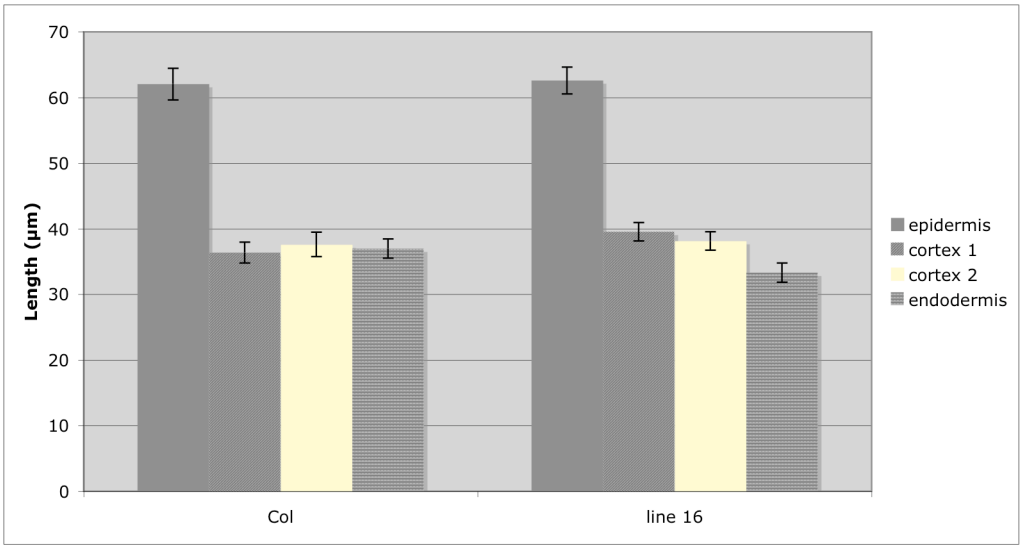


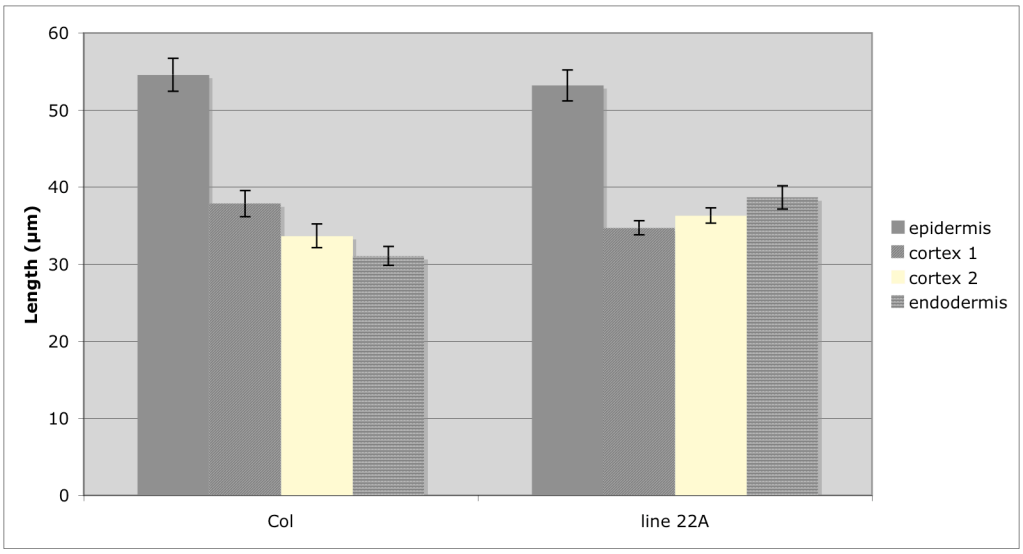
Figure 15. Cell length in the middle of the hypocotyls in light condition at day 3 (A), and 6 (B) in XTH16 knockout line and Wt- Col. Error bars represent SE Values are the means of 25 measurement / day/genotype

Line XTH16 showed reduction in cell length in all cell layers at day 3, but there was no significant difference from the WT at day 6 (Fig. 15). In addition, the hypocotyl

length was slightly reduced compared to the wild type although this line did not show any reduction in transcript level (Fig. 11).

XTH22A

A



B

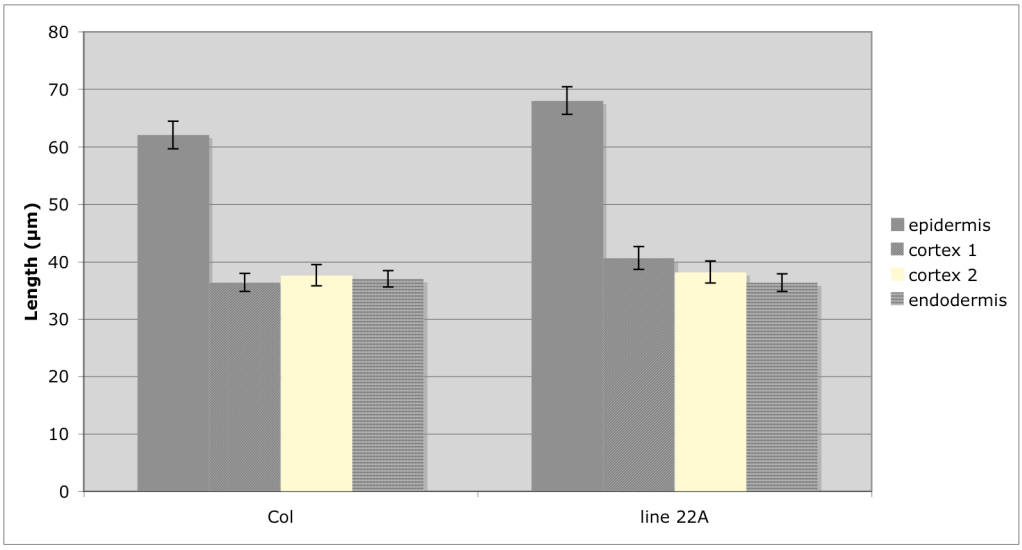


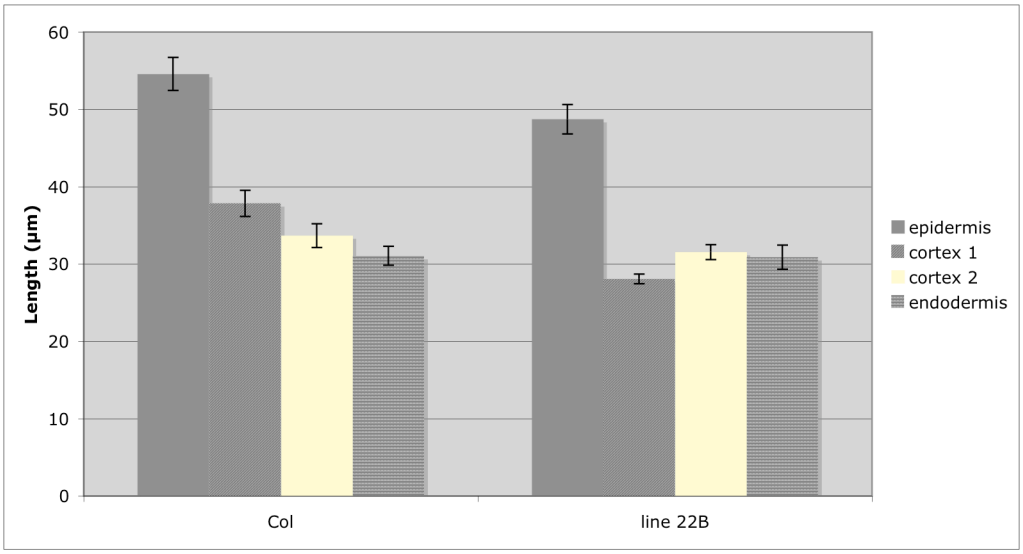
Figure 16. Cell length, in the middle of the hypocotyls, in light condition at day 3 (A), and at day 6 (B) in knockout line XTH22A and WT-Col. Error bars represent SE. Values are the means of 25 measurement/day/genotypes. Values are the means of 25 measurement / day/genotype

In line XTH22A, endodermis cells were longer at day 3, and epidermis and cortex 1 cells were longer at day 6 (Fig. 16). This result is opposite to the theory of an simplastic growth but we know that some cells divisions occurs in the epidermis layer, and

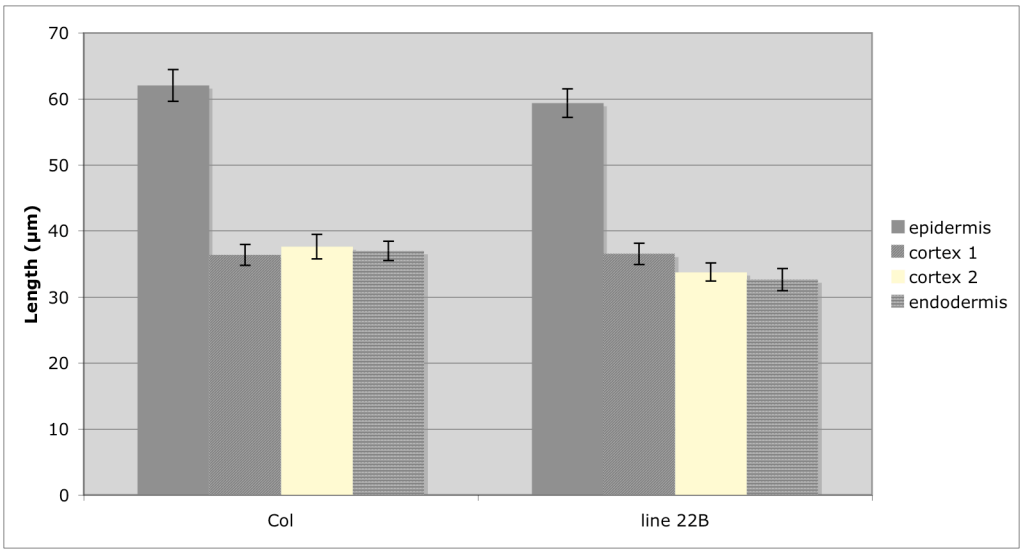
consequently those variations of cell length can be explained by the presence of cell divisions in different layers. The hypocotyl lengths were greatly reduced in this line compared to the wild type in days 3 and 6 (Fig. 11) while cells length was affected only slightly.

XTH22B

A



B



B

Figure 17. Cell length in the middle of the hypocotyls in light condition at day 3 (A), and at day 6 (B) in knockout line XTH22B and WT-Col. Error bars represent SE. Values are the means of 25 measurement / day/genotype

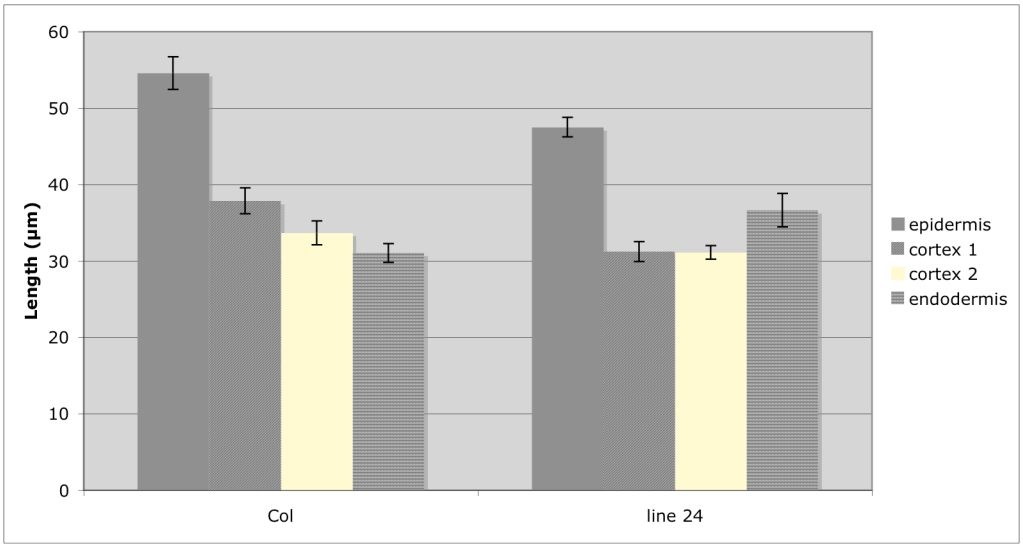
In line XTH22B, cells of epidermis and cortex were smaller at days 3 and 6 and endodermis cells were smaller at day 6 (Fig. 17). Generally the difference between cell

length in XTH22B and WT was not big, and more significant at day 3 than 6 (Fig. 17). Hypocotyls of this line were visibly shorter (Fig. 11).

The results with these two lines, XTH22A and XTH22B, grown in the light would suggests that XTH22 protein is needed for hypocotyl elongation in the light to stimulate cell division either during the embryonic or post-embryonic growth.

XTH24

A



B

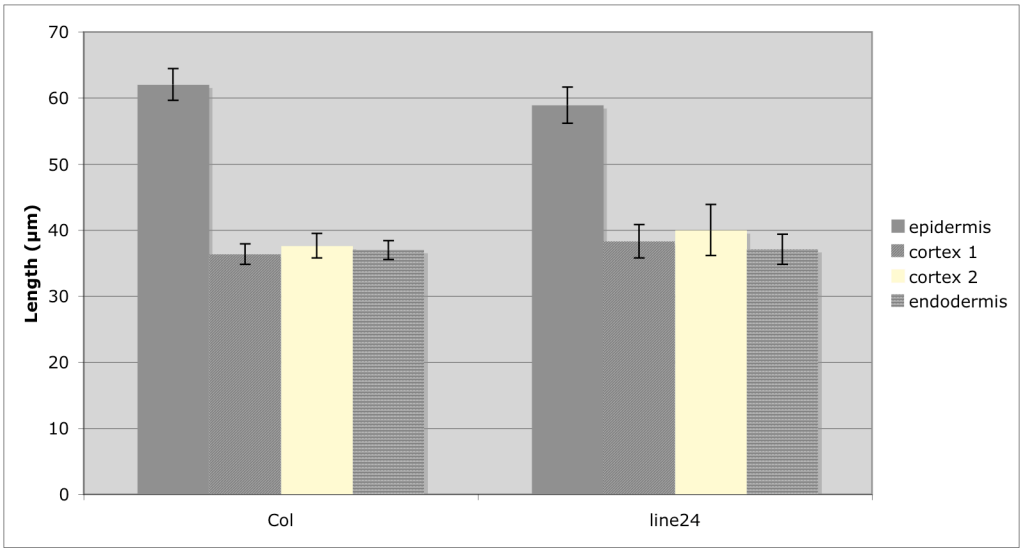


Figure 18. Cell length in the middle of the hypocotyls in light condition at 3 days (A), and at 6 days (B) in XTH24 knockout line and WT-Col. Error bars represent SE. Values are the means of 25 measurement / day/genotype

In line 24, cells of epidermis and cortex were shorter while endodermis cells were longer at day 3 (Fig. 18A). At day 6, no changes in cell lengths were observed (Fig. 18B). The hypocotyl length in this line was greatly inhibited compared to the wild type (Fig. 11).

In summary, the cell lengths were not much affected, even sometimes increased, in all XTH mutants analyzed in the light at day 3 and 6. In contrast, the hypocotyl lengths were significantly affected. This indicates that these mutants had fewer cells and therefore must have been affected in cell division in the hypocotyl.

d. Time course of hypocotyl elongation in the dark condition

There was no significant change in hypocotyl elongation in the dark in the knockout lines compared to the wild type plants (Fig. 19).

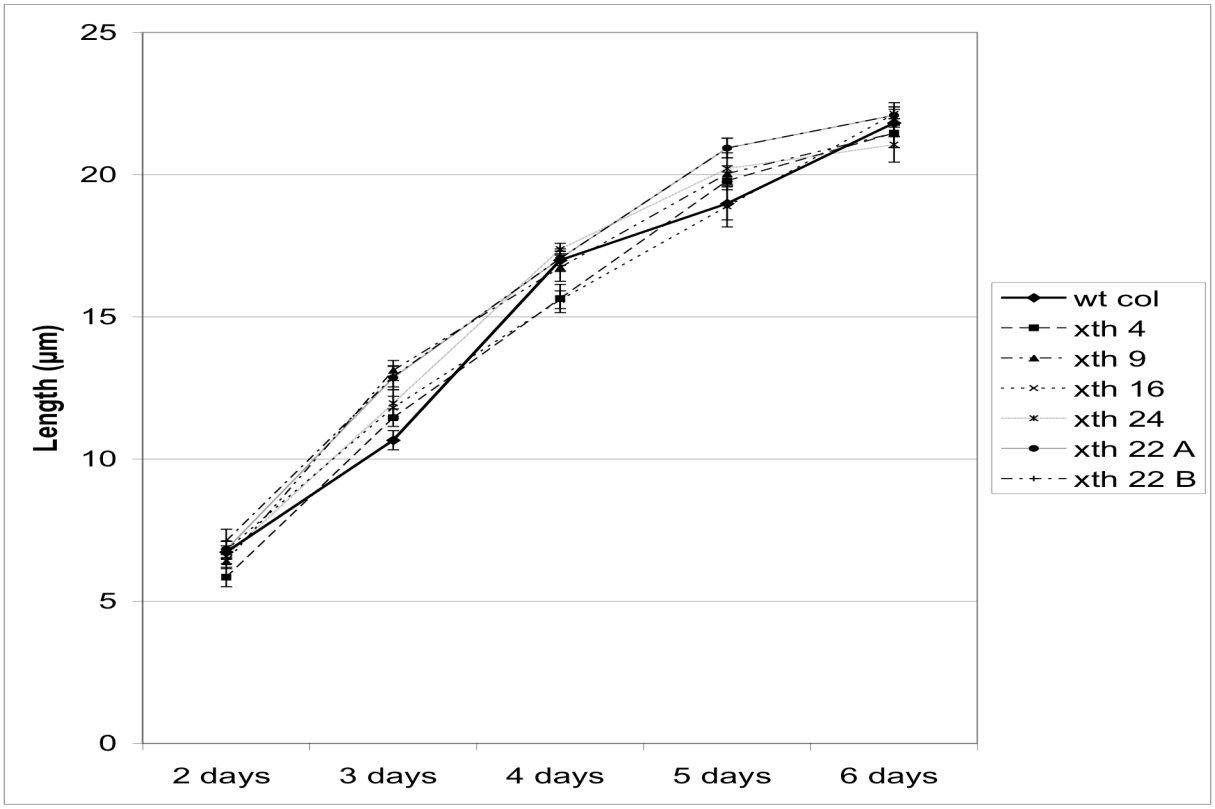


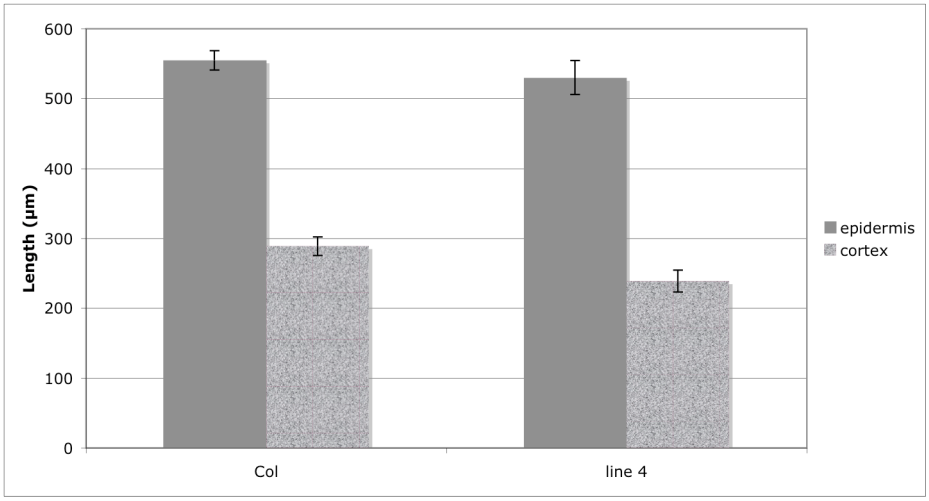
Figure 19. Time course of hypocotyl elongation in the knock-out XTH lines growing in the dark condition. Error bars represent SE. Values are the means of 10 measurement / day/genotype

e. Effects knock out mutations on the cell size in different cell layers of dark-grown seedlings

In line XTH4, epidermis cells were shorter at day 3 but not at day 6 (Fig. 20). The cortex cells were shorter at day 3 and longer at day 6 compared to the wild type. Endodermis cells were not measured because they could not be visualized. Thus the number of cortex cells of the knock out mutant XTH4 appears to be reduced.

XTH4

A



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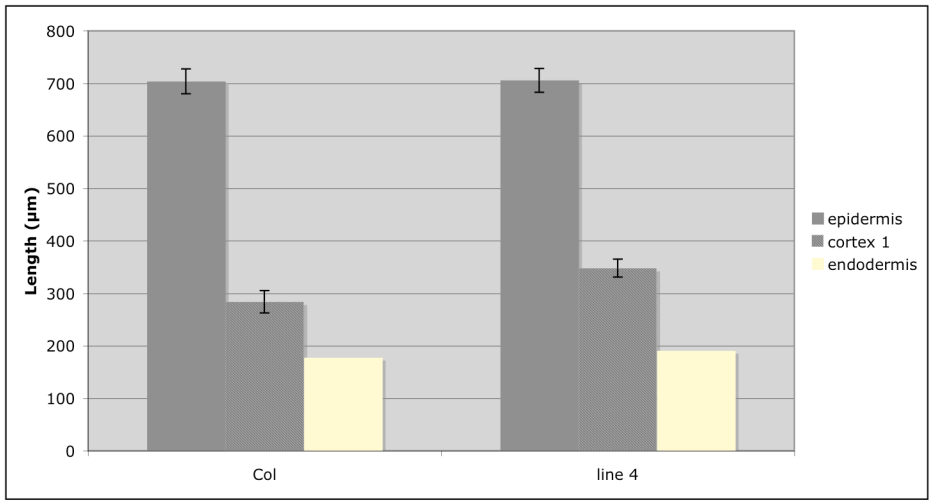
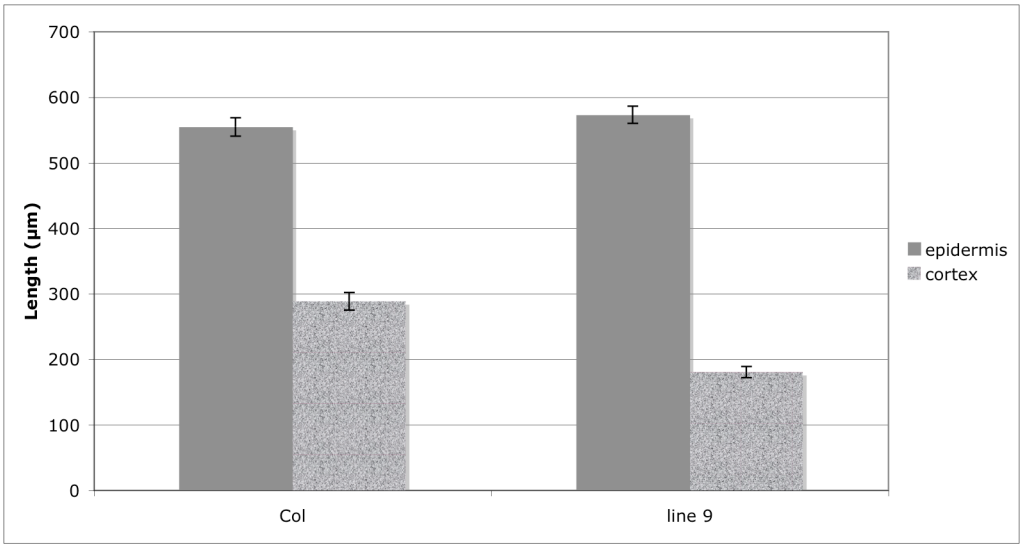


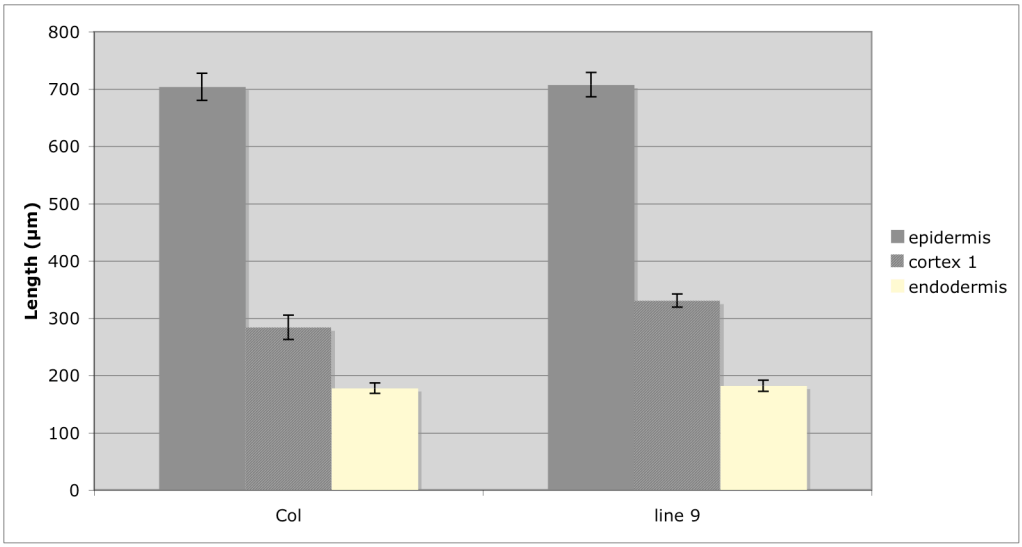
Figure 20. Cell length in the middle of the hypocotyls in dark condition at 3 days (A), and 6 days (B) in XTH4 knockout line and WT-Col. Error bars represent SE. Values are the means of 25 measurement / day/genotype

XTH9

A



B



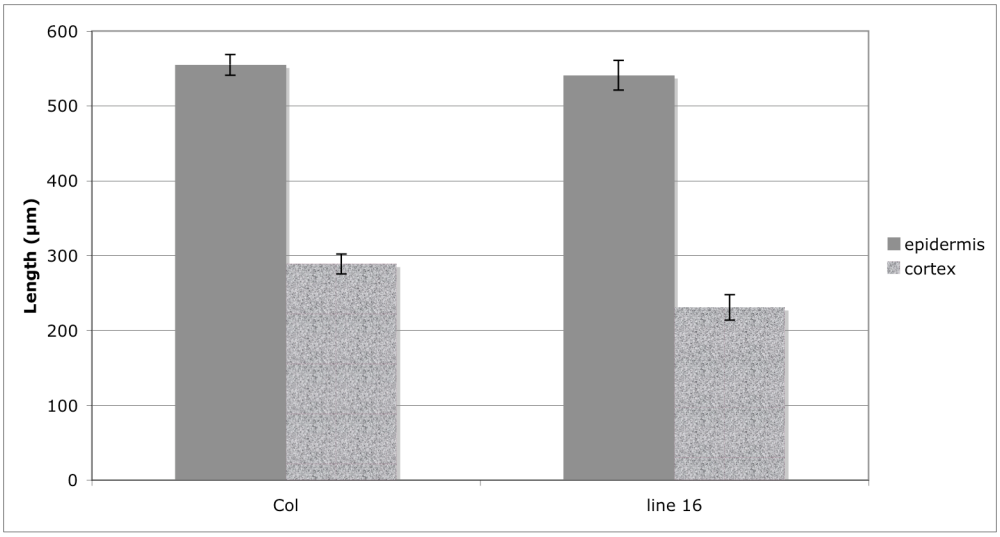
B

<u>Figure 21</u>. Cell length in the middle of the hypocotyl in dark condition at day 3 (A), and 6 (B) in knockout XTH9 line and WT-Col. Error bars represent SE. Values are the means of 25 measurement / day/genotype.

In line XTH9, all cell sizes were similar to the wild type, except for the cortex cells at day 3, which were shorter at day 3 and longer at day 6 (Fig. 21). This might reflect variability along the hypocotyl length.

XTH16

A



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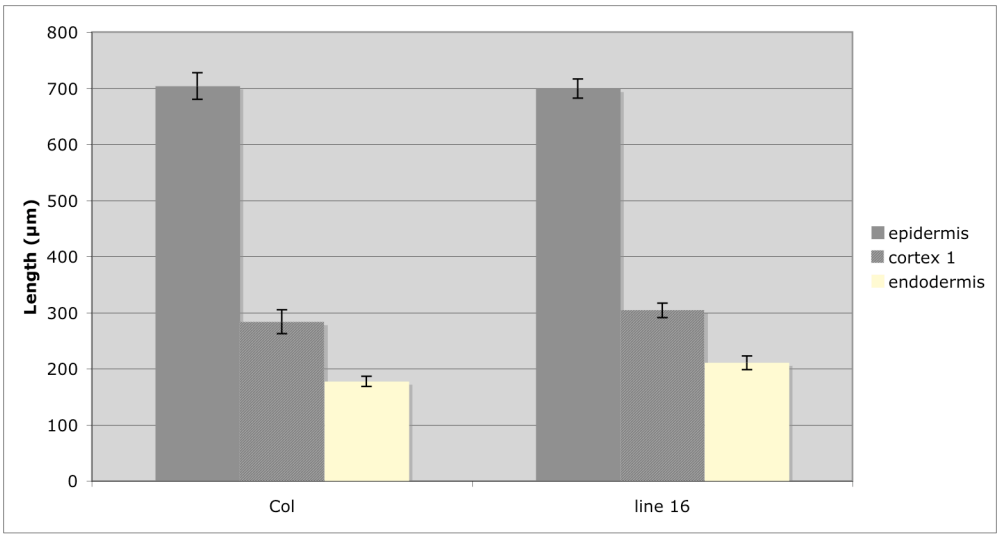
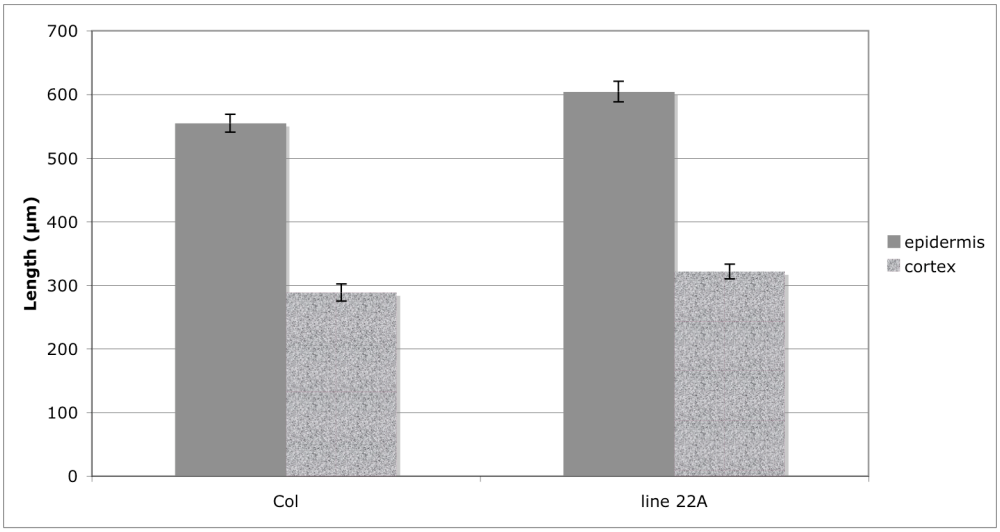


Figure 22. Cell length, in the middle of the hypocotyls, in dark condition at 3 days (A), and at 6 days (B) in XTH knockout lines 16 and WT-Col. Errors bars represent SE. Values are the mean of 25 measurement / day/genotypes

In line XTH16, the only significant change in cell length observed was a slight reduction in cortex cell length at day 3 (Fig. 22). This is also likely due to the variation along the hypocotyl.

XTH22A

A



B

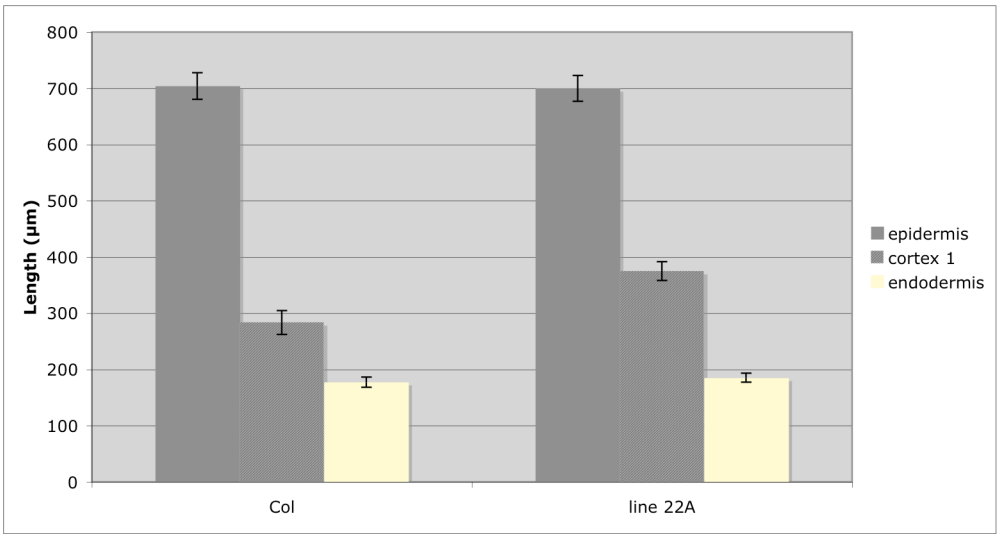
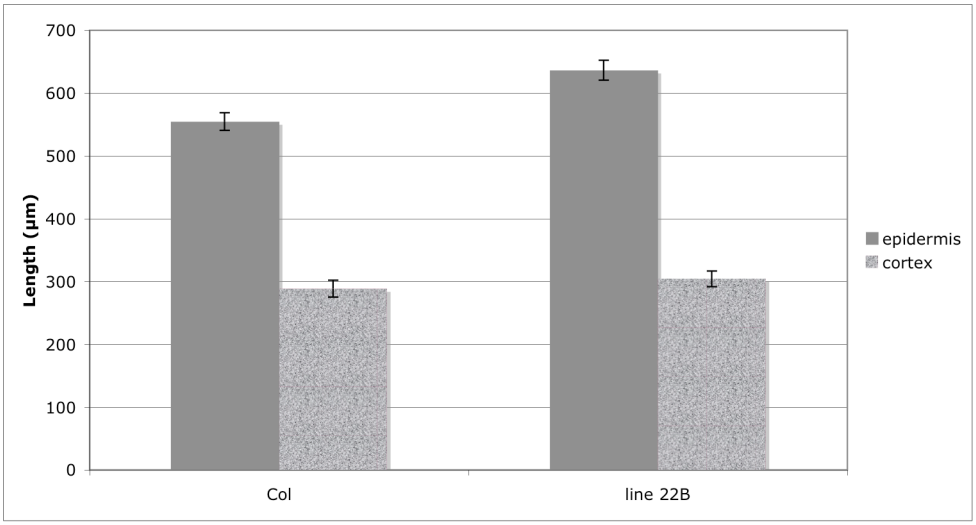


Figure 23. Cell length in the middle of the hypocotyls, in dark condition at day 3 (A), and 6 (B) in XTH knockout line 22A and WT-Col. Error bars represent SE. Values are the means of 25 measurement / day/genotype

In line XTH22A, the cortex cells were longer, particularly at day 6 (Fig. 23). There was no visible variation in the length of other cell types.

XTH22B

A



B

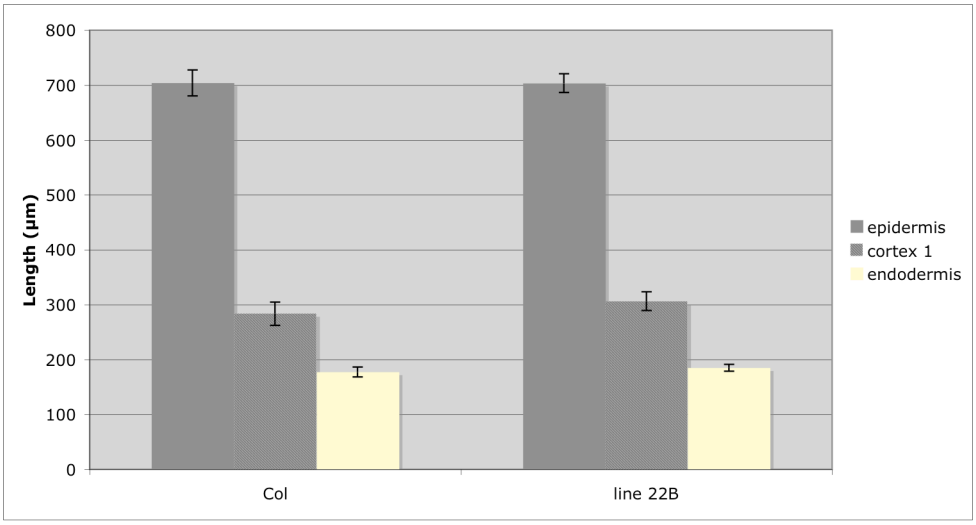
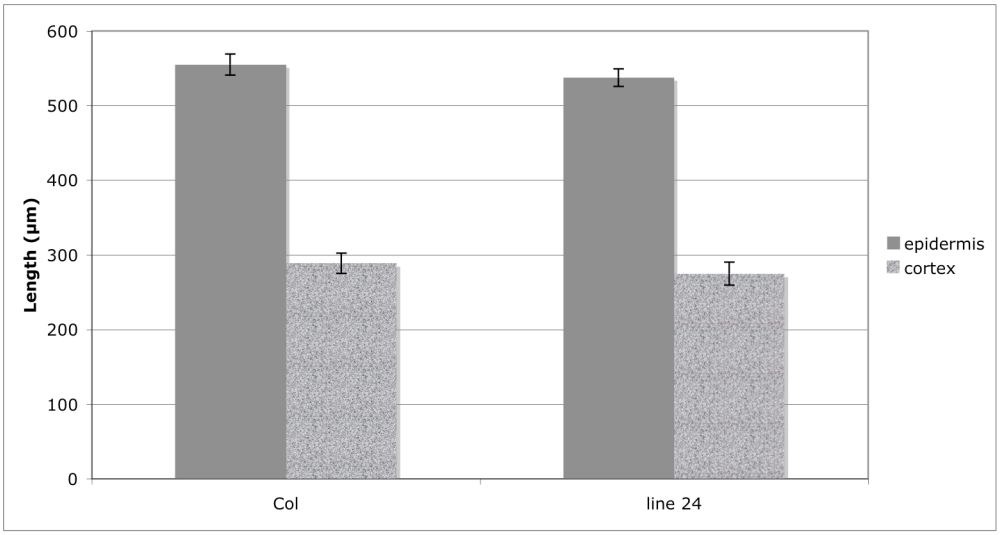


Figure 24. Cell length in the middle of the hypocotyls in dark condition at day 3 (A), and 6 (B) in XTH knockout line 22B and WT-Col. Error bars represent SE. Values are the means of 25 measurement / day/genotype

In line XTH22B, the epidermal cells were initially longer compared to WT but at day 6, this difference disappeared (Fig. 24).

XTH24

A



B

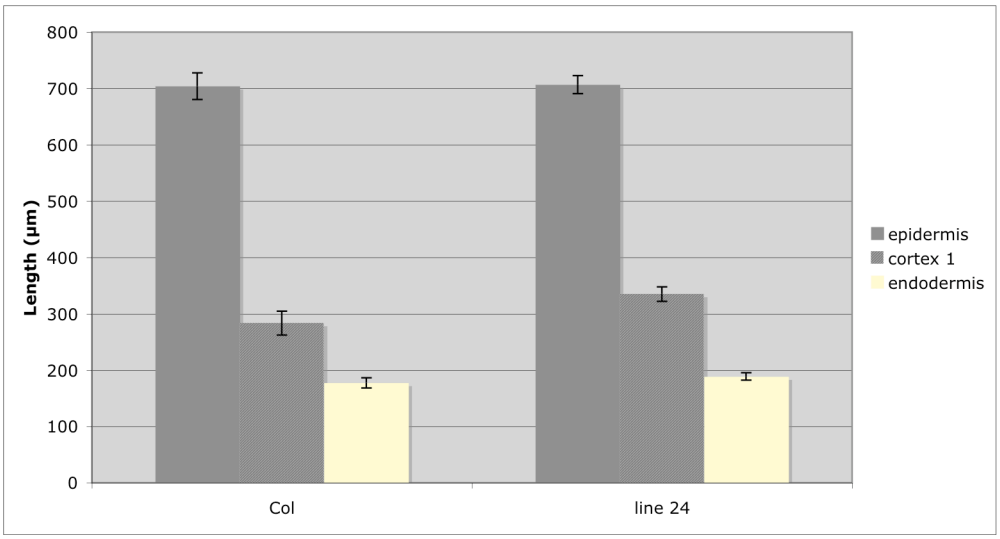


Figure 25. Cell length in the middle of the hypocotyls in dark condition at day 3 (A), and 6 (B) in knockout XTH 24 line and in WT-Col. Error bars represent SE. Values are the means of 25 measurement / day/genotypes

In line XTH24, cortex cells were longer than WT at day 6, cells of the other layers were similar length to the wild type (Fig. 25).

In seedlings growing in the dark no visible differences in the hypocotyl length were observed, but occasionally, a longer cell size in the cortex or endodermis was found. This implied that the mutations affect cell division in these layers.

e. Effects of knock out mutations in the selected XTH genes: discussion

The hypocotyl elongation seems to be inhibited in all the knockout lines in the light, but cell lengths were only marginally affected. In some cell types, there was even an increase in the cell length in the mutant despite of overall growth inhibition. In the dark grown seedlings, there was no change in the hypocotyl elongation but cell length was increased in the cortex in most mutants. This implies that the mutant lines had fewer cells in the cortex and perhaps also in other cell layers. Since similar observations were made on the light and in the dark grown seedlings, it is likely that the mutations in XTH genes affect cell divisions in the embryonic stage of growth.

4. Effects of mutations in KORRIGAN gene and ectopic expression of PttCel9B on hypocotyl elongation

a. Description of mutant lines used in the study

Three alleles of mutated *KORRIGAN* genes were used, *kor1-1* in the Ws background, which is a strong mutation (Sato et al., 2001), and two less severe mutations, *irx2-1* in the *Ler* background and *irx2-2* in the *Col* background. For *irx* lines, collapsed vessel phenotype was reported (Szyjanowicz et al. 2004).

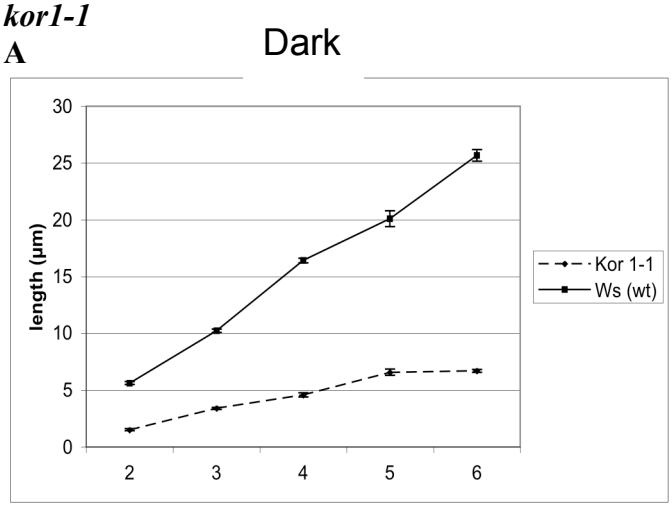
PttCel9B encodes an endoglucanase that is highly expressed in poplar dividing and expanding secondary xylem (Junko Takahashi et al, in preparation). Single insert *35S::PttCel9B* lines were obtained and homozygotic lines were used in this study.

b. Time course of hypocotyls elongation in plants with mutations in KORRIGAN gene

In the dark, *kor1-1* had shorter hypocotyls, but in the light hypocotyls of the mutant and WT had similar length (Fig. 26).

kor1-1

A



B

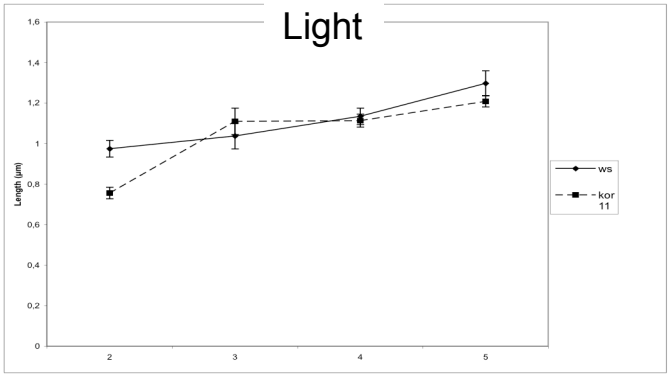
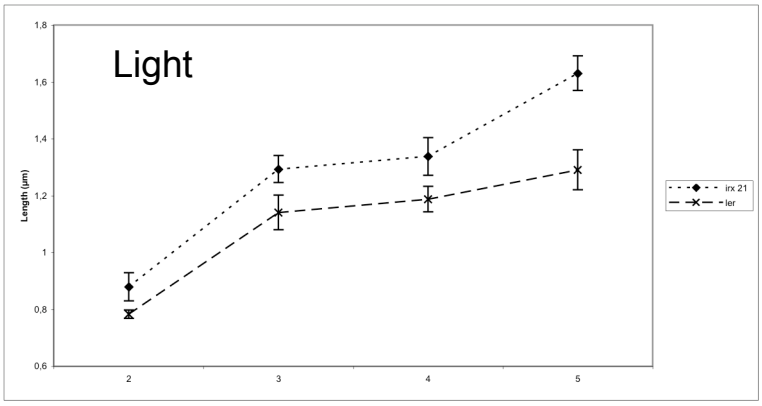


Figure 26. Time-course of hypocotyl elongation in *kor1-1* mutant and the WT-Ws, in the dark (A) and in the light (B) conditions. Error bars represent SE. Values are the means of 10 measurement / day/genotype.

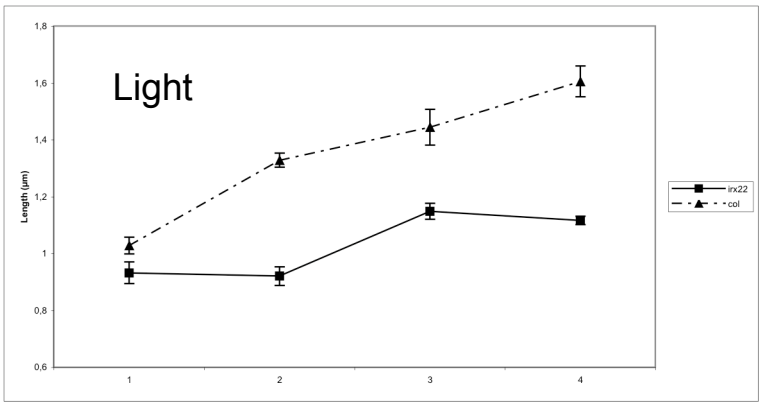
A

irx2-1



B

irx2-2



C

irx2-2

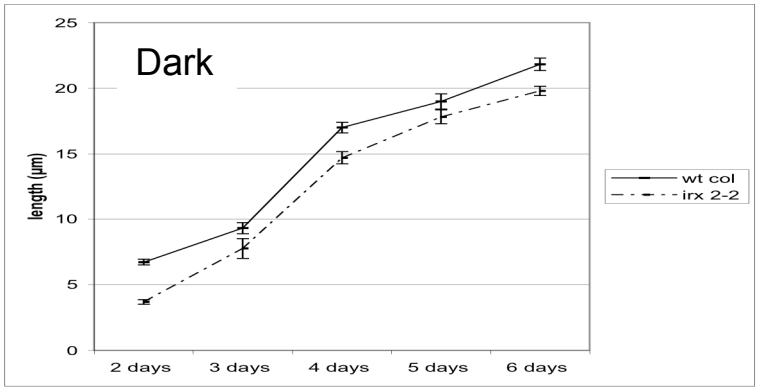


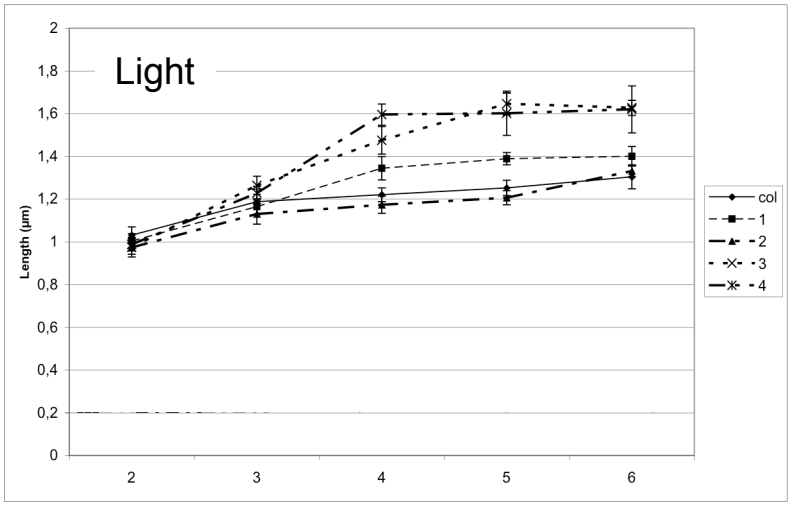
Figure 27. Time course of hypocotyl elongation of *irx 2-1* in the light (A) and of *irx 2-2* in the light (B) and in the dark(C). Values are the mean of 10 measurement / day/genotype

irx2-2 allele induced shorter hypocotyls in the dark but the effects were not as severe as in *kor1-1* (compare Fig. 26A and Fig. 27C). *Irx2-1* allele induced shorter hypocotyls in the light, which was not the case of *kor1-1 or irx2-2* (compare Fig. 26B and 27AB).).

This result confirms that KORRIGAN endoglucanase is needed for growth, and its absence could increase wall rigidity. Different effects were observed in the light are at present difficult to explain. However, the expectation was that dark condition exaggerates the phenotype because of a higher rate of growth. This appears to be the case.

c. Time course of hypocotyl elongation in PttCel9B overexpressing lines

A



B

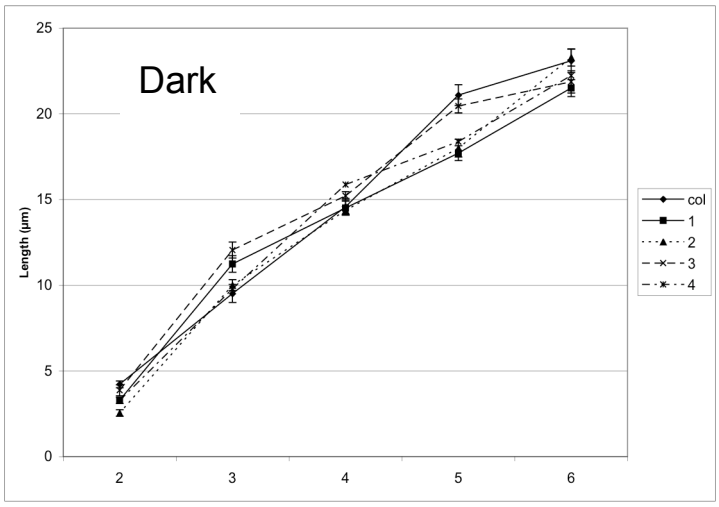


Figure 28. Time-course of hypocotyl elongation in WT and different lines with ectopic expression of PttCel9B in the light (A) and in the dark (B) condition. Error bars represent SE. Values are the means of 10 measurement / day/genotype.

In the dark condition transgenic lines with overexpression PttCel9B did not significantly differ from the wild type in hypocotyl elongation (Fig. 28B). While in the light all lines except line 2 showed an increase of the hypocotyl length (Fig. 28A).

These results suggest that *PttCel9B* activity does not limit dark elongation but can increase hypocotyl elongation in the light. This cellulase seems to play an essential function and it could induce cell expansion.

e. Effects of mutations in KORRIGAN gene and ectopic expression of PttCel9B on hypocotyl elongation: discussion

In the dark condition *kor1-1* and *irx2*-2 mutants induced shorter hypocotyls in comparison to the WT. This result confirms that this endoglucanase can weaken cell wall, and its absence could increase wall rigidity. But *irx2-1* did not show similar inhibition. Perhaps the difference could be explained by a difference of background genotype.

In *PttCel9B* overexpressing lines, the hypocotyl elongation was significantly increased in the light condition. These results indicate that *PttCel9B* similar to KORRIGAN induce cell expansion. Effects of mutations are more severe in the dark. In contrast, the effects of overexpression were observed more distinctively in the light. It is possible that different cellulases have important function in different growth conditions.

VI CONCLUSION

One of the aims of these experiments was examination the growth dynamics and its distribution in *Arabidopsis* hypocotyls of three ecotypes in the light and dark condition. These results were used as background information for subsequent experiments. The second – major aim was to determine the putative involvement of selected members of XTH and cellulase families in hypocotyl growth.

Three ecotypes of *Arabidopsis* (Co, Leer and Ws) were different in hypocotyl elongation. Most striking was the difference between Ws and Col growing in the light condition, with Col having three times shorter hypocotyls. I found that hypocotyl completes its elongation within 6 days in the dark and 5 days in the light in the experimental conditions. Dark grown hypocotyls could not further elongate when transferred to light after 6 days of growth. The rate of growth of hypocotyls was between 2 to 4 times faster in the dark compared to light and it continued for one day longer. The final hypocotyl length was correspondingly much more in the dark.

Epidermis had the most elongated cells in comparison with cortex 1, cortex 2, and endodermis. The sub-epidermal cell layers were not much different in length in general. The non-uniform elongation of different cell layers along the hypocotyl could be explained by the possibility of different rates of cell divisions in some layers. Most of these divisions would be completed during embryonic growth, but some cell layers might have more divisions than others. Epidermis could have also divisions related to the formation of stomatal complexes during germination in the light.

For most *PttXET16A* lines, stronger *PttXET16A* expression coresponded to more growth inhibition and shorter hypocotyls. Generally, cell length was also inhibited, but there were several exceptions of lines and cell layers either not affected, or even stimulated in length. Consequently, I could deduce that many overexpressing lines had fewer cells in some cell layers. Those results should be verified by independent means, but they do suggest that the inhibition of hypocotyl elongation by *PttXET16A* may operate at two different levels, first at the level of cell division and second at the level of cell expansion. Probably PttXET16A enzyme reduces cell wall plasticity by incorporation of xyloglucan to the wall and thus creating a possibility for more xyloglucan cross-links between cellulose microfibrils, affecting cell expansion between cell divisions, and during cell differentiation.

Arabidopsis XTHs have been previously identified in the hypocotyls of the plants induced to form a wood. Single locus T-DNA insertions mutants of these genes were obtained. I studied the elongation of the mutant hypocotyls in the light and dark conditions and the effects on the cell sizes. The mutants had much reduced hypocotyl lengths in the light, but only a weak reduction or sometimes even a stimulation of cell length was observed. Thus the hypocotyls must have had fewer cells. In the dark, no significant change in hypocotyl length was found, but in several cases, an increase of cell length could be seen. Decreasing the hypocotyl length without decreasing the cell length in the XTH mutants also indicates that the mutations affected cell division, most likely in

the embryo. The mechanism of this might be different than in the case of overexpressing lines. I propose that the mutants had weaker, more plastic cell walls, thus more cell expansion was possible between cell divisions during meristematic growth. As a consequence there was a reduction of cell number in the embryos. This prediction should be verified by independent means but it illustrates the point that it is very difficult to draw any conclusions regarding the molecular mechanism of XTH action based on these measurements.

To address the possibility of redundancy, double mutants were created and homozygotic double mutant lines of XTH9 x XTH22, XTH4 x XTH22, XTH4 x XTH9 were isolated for further studies.

Lines with overexpression of cellulase PttCel9B had longer hypocotyls in the light condition. The KORRIGAN cellulase mutant, *kor1-1* had shorter hypocotyls in the dark and *irx2-2* in the light. This result confirms that this endoglucanase can weaken cell wall, and its absence could increase wall rigidity. *irx2-1* mutant did not show consistent results. Studied mutants were made in different backgrounds (*kor1-1* in Ws, *irx2-1* in Lerl and *irx2-2* in Col) which could have contributed to variable reactions. Results from the study suggests that both cellulases *PttCel9B* and KORRIGAN are important for cell expansion.

Hypocotyls are widely used as a test system to study cell expansion. The present results demonstrate the relative complexity of the regulation and the mechanism hypocotyl expansion, and in order to elucidate the action of enzymes as cellulases, xyloglucan endotransglycosylases, and hydrolases, some experiments at later stages of development and studies of mechanical wall properties could be very interesting.

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VI PROTOCOLS

DNA extraction

"Rapid Genomic DNA extraction method"
(Edward et al. Nucleic Acid Research vol. 19 No.6 p1349 1991)

Extraction buffer 200 µM Tris-HCl pH 7.5 250 µM NaCl 25 µM EDTA 0.5% SDS

1. Leaf sample (Ca. 10 100 mg)
2. Grind tissue with hand drill for 15 sec
3. Add 400 µl of extraction buffer
4. Vortex for 5 sec.
5. The mixture can be left at room temperature to 1 hr
6. Spin at 13000 rpm for 5 min.
7. Transfer 300 ul of supernatant to a new tube.
8. Add 300 µm of isopropanol
9. Vortex for 5 sec.
10. Stand at room temperature for 5 min.
11. Spin at 13000 rpm for 5 min.
12. Discard supernatant and add 500 µl 70% Et-OH.
13. Vortex for 5 sec.
14. Spin at 13000 rpm for 5 min.
15. Discard supernatant and dry up the pellet with speed-vac.
16. Dissolve pellet in 50 100 µl TE (50 deg heat block)

PCR

PCRs were performed in 20 µm volume with following concentrations: 1 X Taq polymerase buffer, 200 µm d NTP mix, 0,5µm of each primer, x of template DNA
Temperature cycling:

- 94^{o}C for 30 sec (denature)
- 56 ^{o}C for 40 sec (anneal)
- 72^{o}C for 50 sec (elongate) (60 sec per kb target sequence length)
- 34 cycles
- 72^{o}C for 5 min at end to allow complete elongation of all product DNA

Seedling clearing and staining protocol

Reference: Malamy J E, Benfey P N (1997)

1- Seedling were incubated in Petri dish containing 0, 24 N HCl in 20% methanol at 57% on heating plate for 15 minutes.
2- Remove the Petri dish off the heating plate, and gently pipette out the acid solution without damaging seedlings. Replace first clearing solution with adding and removing (three times) second clearing solution with Pasteur pipette. Second clearing solutions consist in 7% NaOH, in 60% ethanol.
3- Congo red a concentrated 0,1% solution can be added as few drops at this stage, but can be also added at the first step of dehydration of the seedling.
4- Dehydration was carried out by changing the series of ethanol solution (starting with 40%, than 20%, followed by 10%) and incubating seedlings for 5 minutes in each solution. The dehydration series are also allowing for removal of excess of Congo red from media. The seedlings were vacuum unfiltered for 15 minutes with final solution of 25% glycerol in 5% ethanol.
5- Store seedlings in this solution.
6- Whole seedlings were mounted on 50% glycerol on the microscope slides and enclosed with cover slips for observation.