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# Root Hair Initiation Is Coupled to a Highly Localized Increase of Xyloglucan Endotransglycosylase Action in Arabidopsis Roots<sup>1</sup>

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Root hairs are formed by two separate processes: initiation and subsequent tip growth. Root hair initiation is always accompanied by a highly localized increase in xyloglucan endotransglycosylase (XET) action at the site of future bulge formation, where the trichoblast locally loosens its cell wall. This suggests an important role of XET in the first stages of root hair initiation. The tip of growing root hairs is not marked by localized high XET action. Experiments in which root hair initiation was modulated and observations on root hair mutants support this view. The ethylene precursor 1-aminocyclopropane-1-carboxylic acid shifts both root hair initiation and the local increase in XET action toward the root tip. On the other hand, roots treated with the ethylene inhibitor aminoethoxyvinyl-glycine, as well as roots of mutants affected in root hair initiation (*rhl1*, *rhd6-1*, and *axr2-1*) revealed no localized increases of XET action at all and consequently did not initiate root hairs. Disruption of actin and microtubules did not prevent the localized increase in XET action. Also, the temporal and spatial pattern of action as the specific pH dependence suggest that different isoforms of XET act in different processes of root development.

Root hairs are tip-growing tubular extensions of root epidermal cells. They play an important role in the uptake of water and nutrients and in anchoring the root in the soil (Peterson and Farquhar, 1996) and in some species also serve as the interface between the plant and a variety of fungal or bacterial symbionts (Peterson, 1992). It is known that sequential expression of different genes is necessary to obtain the finger-shaped outgrowth known as the root hair (Parker et al., 2000; for review, see Schiefelbein, 2000). Root hairs emerge from a subset of specialized epidermal cells called "trichoblasts" (Leavitt, 1904).

In Arabidopsis the patterning of hair-forming and non-hair-forming cells is highly regulated, i.e. epidermal cells that lie over a junction between a pair of cortical cells assume the characteristics of trichoblasts; the cells overlying single cortical cells become atrichoblasts (e.g. Dolan et al., 1994; Galway et al., 1994).

Besides positional information, genes such as *TRANSPARENT TESTA GLABRA (TTG)* and *GLABRA2 (GL2)* are important as they appear to code for

negative transcriptional regulators of root hair formation (Galway et al., 1994; Di Christina et al., 1996; Masucci et al., 1996; for review, see Gilroy and Jones, 2000; Schiefelbein, 2000). The fate of an epidermal cell may be determined by the relative abundance of CAPRICE (CPC), a negative regulator of GL2 (Wada et al., 1997), and WEREWOLF (WER), a positive regulator of GL2 transcription (Lee and Schiefelbein, 1999; for review, see Schiefelbein, 2000).

Mutants such as *ctr1*, *rhd6*, and *axr2* also indicate the possible involvement of hormone-related mechanisms (especially auxin and ethylene) in cell fate specification (Wilson et al., 1990; Kieber et al., 1993; Masucci and Schiefelbein, 1994; Tanimoto et al., 1995, 1996).

Trichoblasts are highly polar during their elongation. The initiation of root hairs requires the establishment of a new type of cell polarity, while maintaining the previous polarity (Le et al., 2001). The trichoblast then locally loosens the cell wall and undergoes highly localized expansion at its outer surface to form a bulge in the cell wall (Leavitt, 1904). Once initiated, tip growth starts and cell wall deposition is confined to the expanding tip of the growing hair, leading to the elongated hair-like outgrowth (Schnepf, 1986). In wild-type Arabidopsis plants, this bulge in the trichoblast wall is always formed at the apical end of the cell (the end nearest the root tip; Schiefelbein and Somerville, 1990). Root hair initiation, seen as bulging of the cell wall, is associated with microtubule and actin reorganizations in the cytoplasm (Emons and Derksen, 1986; Baluška et al.,

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2000b) and is causally linked with local acidification in the cell wall (Bibikova et al., 1998). However, other factors than acidification alone must define the precise site of outgrowth of the root hair since artificial acidification of the entire trichoblast wall does not alter the site of bulge formation.

Bulge formation requires highly localized cell wall modifications. Among several potential cell wall-modifying enzymes (Fry, 2000), xyloglucan endotransglycosylase (XET) could account for localized rearrangement of tethers in the apoplast (Fry et al., 1992). XETs are enzymes that cleave and rejoin xyloglucan chains (Baydoun and Fry, 1989; Smith and Fry, 1991; Fry et al., 1992; Nishitani and Tominaga, 1992; Lorences and Fry, 1993; Thompson and Fry, 2001), and may thereby reversibly loosen the cell wall. In this polysaccharide-to-polysaccharide transglycosylation reaction, one xyloglucan molecule acts as the donor substrate and a second one acts as the acceptor substrate. Because the products need not differ chemically from the substrates, the reaction on endogenous substrates is difficult to detect *in vivo*; progress has relied on experiments using dual labeling ( $^{13}\text{C}/^{13}\text{H}$ ) of endogenous xyloglucans (Thompson et al., 1997; Thompson and Fry, 2001).

It is experimentally convenient that XETs can also utilize xyloglucan oligosaccharides (XGOs) as the acceptor (but not donor) substrate (Baydoun and Fry, 1989; Smith and Fry, 1991; Fry et al., 1992; Nishitani and Tominaga, 1992; Lorences and Fry, 1993), a property widely exploited in the quantitative assay of XET activity, e.g. after extraction of the enzyme from the tissue. XET action (on endogenous substrates *in situ*), as distinct from XET activity (measured in the presence of arbitrary concentrations of exogenous substrates), can be demonstrated using a technique in which exogenous fluorescently labeled XGOs become incorporated into the cell wall at sites where XET acts upon xyloglucan (as donor substrate; Ito and Nishitani, 1999; Vissenberg et al., 2000). Although the fluorescent acceptor substrate is exogenous, both the enzyme and the donor substrate are endogenous and the formation of the fluorescent polymeric products therefore provides evidence for the action of the enzyme on an endogenous substrate *in situ*, as opposed to demonstrating the mere presence of (potentially) active enzyme.

Using a new fluorescent technique, we have previously shown that in cells of the diffuse growth type, high XET action is specifically confined to actively elongating (Vissenberg et al., 2000) or expanding cells (Verbelen et al., 2001). Here, we report that XET action is increased at the site of root hair initiation just before and during the onset of bulge formation, whereas during typical tip growth XET is active all over the surface of the root hair. The role of XET in root hair initiation and subsequent tip growth is further discussed.

## RESULTS

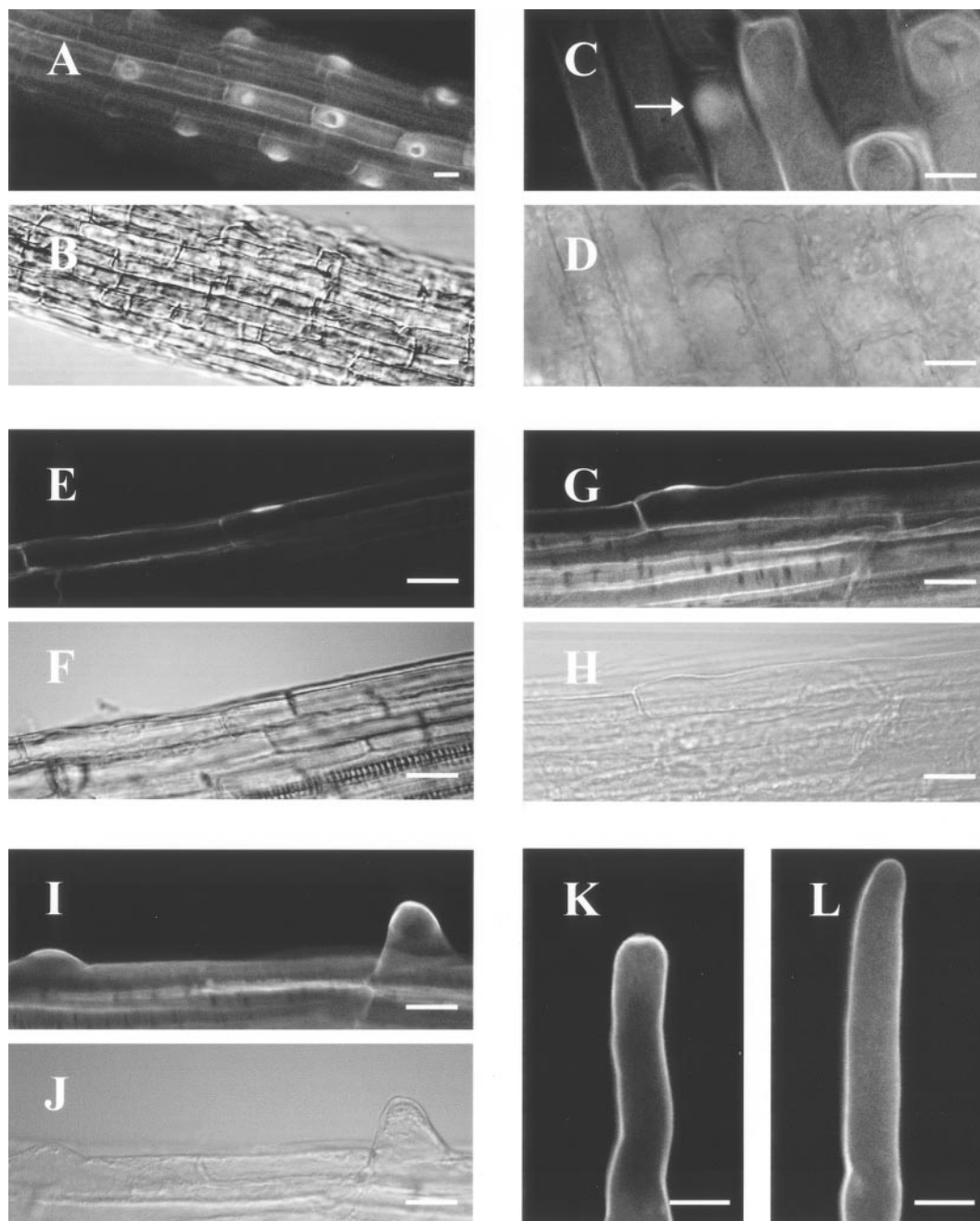
We used the technique described by Vissenberg et al. (2000) to visualize XET action at the moment of initiation and during further tip growth of root hairs in *Arabidopsis*. Fluorescence on the confocal pictures is due to the incorporation of sulforhodamine-labeled oligosaccharides of xyloglucan (XGO-SRs). For clarity, root tips are always pointing to the left side of the pictures except in Figure 1, C and D, where the root tip points to the upper side.

### High XET Action Delineates Sites of Root Hair Initiation

In a surface view of the differentiation zone of a root, emerging root hair bulges are highly fluorescent. This is seen as fluorescent patches and ring structures on the less fluorescent background of the root surface (Fig. 1A). The corresponding bright-field picture can be seen in Figure 1B. At higher magnification (Fig. 1, C and D), fluorescent patches can already be detected in trichoblast walls before any bulging is visible. The arrow in Figure 1C points to such a spot on a trichoblast surface. The ring patterns seen in Figure 1, A and C, are in fact artifactual representations of plain fluorescent emerging root hair bulges, if only the base of the bulge is included in the optical section, or after its tip has been indented by applying a coverslip. The fluorescence of a complete bulge can be seen for example in the root hairs in the trichoblast cell file at the lower side of the root in Figure 1A and in the roots in Figure 5.

The highly fluorescent area of the wall can also be detected in thin confocal longitudinal sections through trichoblasts at the future site of root hair outgrowth (Fig. 1E), clearly before any sign of bulge formation is visible on the corresponding bright-field picture (Fig. 1F). Subsequent fluorescence micrographs (Fig. 1, G and I), accompanied by the respective bright-field pictures (Fig. 1, H and J), illustrate further phases of root hair initiation and elongation. In every picture, the fluorescence intensity at the bulge area is higher than in the remainder of the trichoblast. In longer root hairs that are still actively elongating, XET action appears approximately uniform all over the surface (Fig. 1, K and L).

As controls, roots were incubated with cellobiose-SR or cellotetraose-SR, both of which are not acceptor substrates for XET (Fig. 2, A and B). These roots displayed no appreciable fluorescence compared with XGO-SR-labeled roots. Inactivation of XET by boiling led to the complete loss of fluorescence (results not shown; for figures, see Vissenberg et al., 2000).

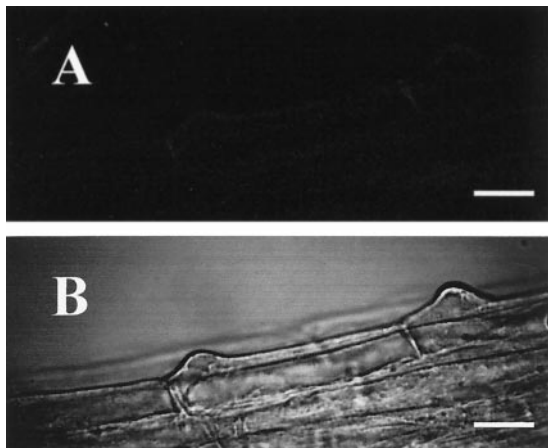


**Figure 1.** Fluorescent XET action donor substrate colocalization during initiation and subsequent growth of root hairs in wild-type Arabidopsis. A, Part of a root showing trichoblasts with high XET action at the site of future root hair outgrowth and in root hair bulges. C, Top view picture of emerging root hairs assayed for XET. The arrow points to a site with high XET action before root hair outgrowth. E, Side view confocal section through a trichoblast clearly demonstrates a highly localized XET action at the future site of root hair emergence. G and I, High XET action in further phases of root hair growth. In all pictures high action is seen at the site of outgrowth. B, D, F, H, and J, Bright-field pictures of the roots in A, C, E, G, and I. K and L, XET assay in older root hairs shows a homogenous distribution of XET action throughout the cell wall. Bars = 20  $\mu\text{m}$ .

#### 1-Aminocyclopropane-1-Carboxylic Acid (ACC) and Aminoethoxyvinyl-Gly (AVG) Modulate the Site of Root Hair Initiation and of High XET Action

The position of root hair formation can be modulated with exogenous hormones and inhibitors and is affected in hormone mutants. Addition of ACC, a

precursor of the hormone ethylene, reduces cell elongation, but does not interfere with root hair initiation in trichoblasts (Le et al., 2001) and root hairs are found much closer to the root apex. Figure 3, A and B, show a thin confocal section through a line of trichoblasts in an ACC-treated root. The local increases in fluores-



**Figure 2.** Chemical and biological controls for XET action during root hair initiation and outgrowth. A and B, Control assay with cellotetraose-SR, a fluorescent non-XET substrate, shows no significant fluorescence in the bulges compared with the clearly fluorescent patches and bulges in Figure 1, E, G, and H. Fluorescence (A) and bright-field image (B). Bars = 20  $\mu\text{m}$ .

cence intensity are present but difficult to see because the root hairs emerged from cells close to the root tip. These cells already display high XET action throughout the wall (Vissenberg et al., 2000). In the most apical cell (nearest the root tip), fluorescence intensity is slightly higher at the forming bulge (see arrow) than in the rest of the cell. These results were comparable with the situation in the *ctr1-1* mutant that constitutively expresses the ethylene triple response (results not shown). Treatment with 10  $\mu\text{M}$  AVG (an ethylene biosynthesis inhibitor) during 4 to 6 h resulted in the loss of root hair initiation (Fig. 3C). Concomitantly, no specific patches of XET-action could be found (Fig. 3D). The bulges that were formed before the transfer to AVG did not grow out, yet displayed high XET-action comparable with Figure 1, I through H (results not shown). In roots of the *etr1-3* mutant that is insensitive to ethylene, root hairs and high XET action were found further away from the root tip (Fig. 3, E and F). Treatment with the auxin indole-3-acetic acid (IAA) did not change the pattern of root hair formation or the local increase of XET action. It only resulted in longer root hairs (results not shown). In the auxin-resistant (*axr2-1*) mutant, fewer root hairs were formed than in the wild-type plants, as in the trichoblast cell line not all cells differentiate into a trichoblast (Fig. 3G). However, every root hair that was initiated displayed the characteristic site with high XET action at the correct place (Fig. 3H).

#### High XET Action Is Insensitive to Cytoskeleton Disruptors

Disruption of F actin and microtubules using la-trunculin B and oryzalin, respectively, had no effect on root hair initiation. In both cases (Fig. 4, A–D)

fluorescence was clearly higher in the forming bulge than in the remainder of the trichoblast. However, disruption of the F actin resulted in the arrest of root hair outgrowth after initiation (data not shown). Oryzalin had no effect on the outgrowth but root hairs sometimes lost their directionality of growth or initiated a second tip and started branching. At these newly formed growing tips, we never found an increase in XET action (Fig. 4, E and F).

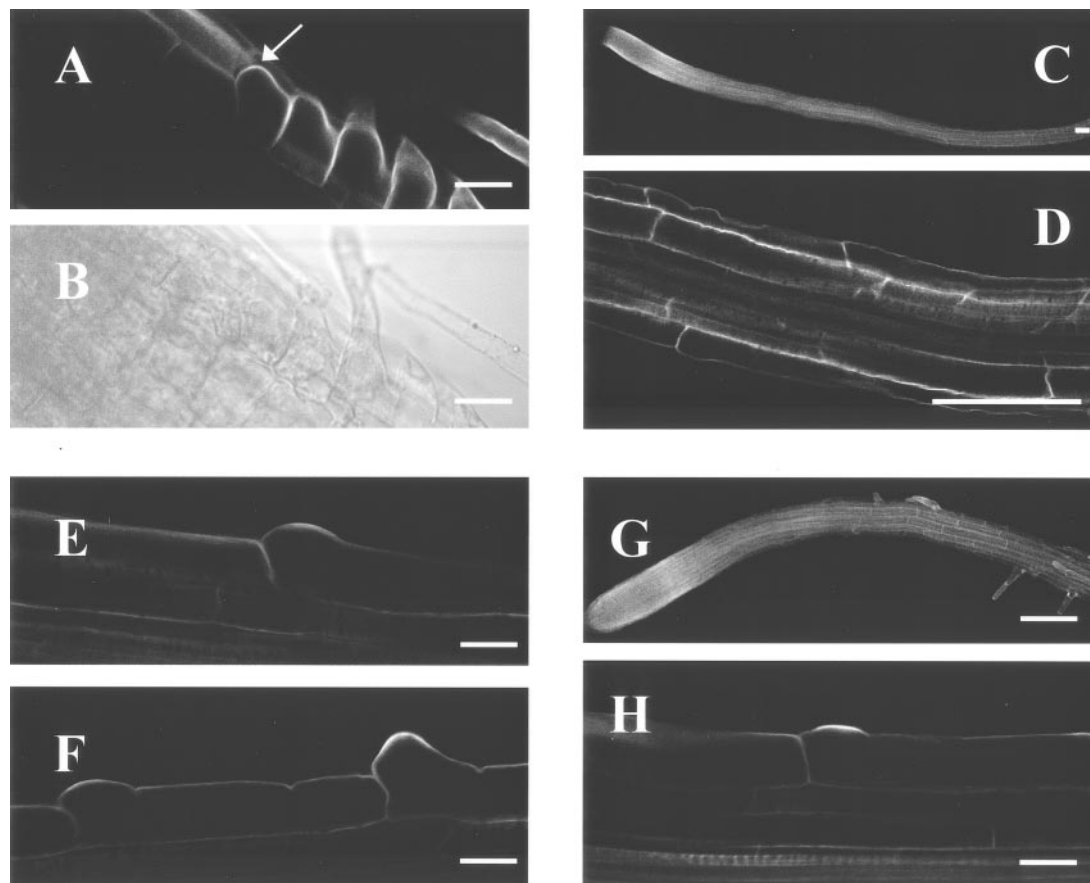
Interference with cellulose deposition using DCB resulted in the swelling of the cells in the elongation zone (as was the case with prolonged oryzalin treatment), yet had no effect on the correct site of root hair initiation. In Figure 4, G and H, higher XET action can be seen in the newly formed bulge.

#### Root Development Involves at Least Three Different XET Actions

The XET action at root hair initiation is specific for its pH dependence. All experiments described above were done at pH 5.5. At this pH, root hair initiation is clearly accompanied by high XET action (Fig. 5, upper root) and the elongation zone also displays high XET action (see also Vissenberg et al., 2000). When the roots were transferred for 6 h to 25 mM MES [2-(*N*-morpholino)-ethanesulfonic acid] at pH 4.5, the high fluorescence in the elongation zone strongly diminished although the growth rate of the root was not affected. The fluorescent root hair bulges were still clearly present (Fig. 5, middle root). On the other hand, when the roots were kept in MES at pH 7.0, root growth was significantly affected (–94% of growth at pH 5.5) and the initiation of new root hairs was inhibited. In the elongation zone, however, high XET action was present. On the contrary, no sign of XET action could be detected in potential trichoblasts (Fig. 5, lower root). The bulges that had been formed before the transfer stopped their development but still displayed high XET action. (results not shown). When the roots were transferred from pH 7.0 back to the medium at pH 5.5, they recovered root hair initiation and the fluorescent spots on trichoblasts caused by XET action reappeared (results not shown).

#### Root Hair Mutants Confirm the Distinct XET Actions at Initiation and during Tip Growth

The data obtained with wild-type plants and with mutants in the ethylene response pathway essentially show that root hair initiation is intimately linked to a local increase in XET action at the site of initiation and occurring before any visible bulge in the wall is found. This strongly suggests that XET action is causally linked to root hair initiation. Because XET action is homogeneously spread throughout the wall of the growing root hair, it seems that this enzyme action is not causally linked to tip growth. This hypothesis



**Figure 3.** Role of ethylene and auxin in the action of XET during root hair initiation and outgrowth in Arabidopsis wild type and mutants. A and B, Root hair initiation and XET action in the elongation zone during ACC treatment. The arrow points to a spot with slightly higher XET-caused fluorescence than in the remainder of the trichoblast. Fluorescence (A) and bright-field image (B). C, Treatment with the ethylene-biosynthesis inhibitor AVG during 6 h inhibits root hair initiation in wild-type roots. High XET action is strictly confined to cells in the elongation zone. D, Detailed picture of XET assay in an AVG-treated root. In potential trichoblasts, no localized up-regulation of high XET-action can be detected (compare with Fig. 1A). E and F, In the ethylene-resistant mutant (*etr1-3*), high XET action can be found in the bulges. G, In roots of the auxin-resistant mutant (*axr2-1*), fewer root hairs are initiated than in wild-type roots. High XET action is only found in cells in the elongation zone and in occasionally formed root hairs. H, When a sporadic root hair is initiated in *axr2-1*, the localized patches with high XET action can be detected. Bars = 20  $\mu\text{m}$ , except in C, D, and G, where bars = 100  $\mu\text{m}$ .

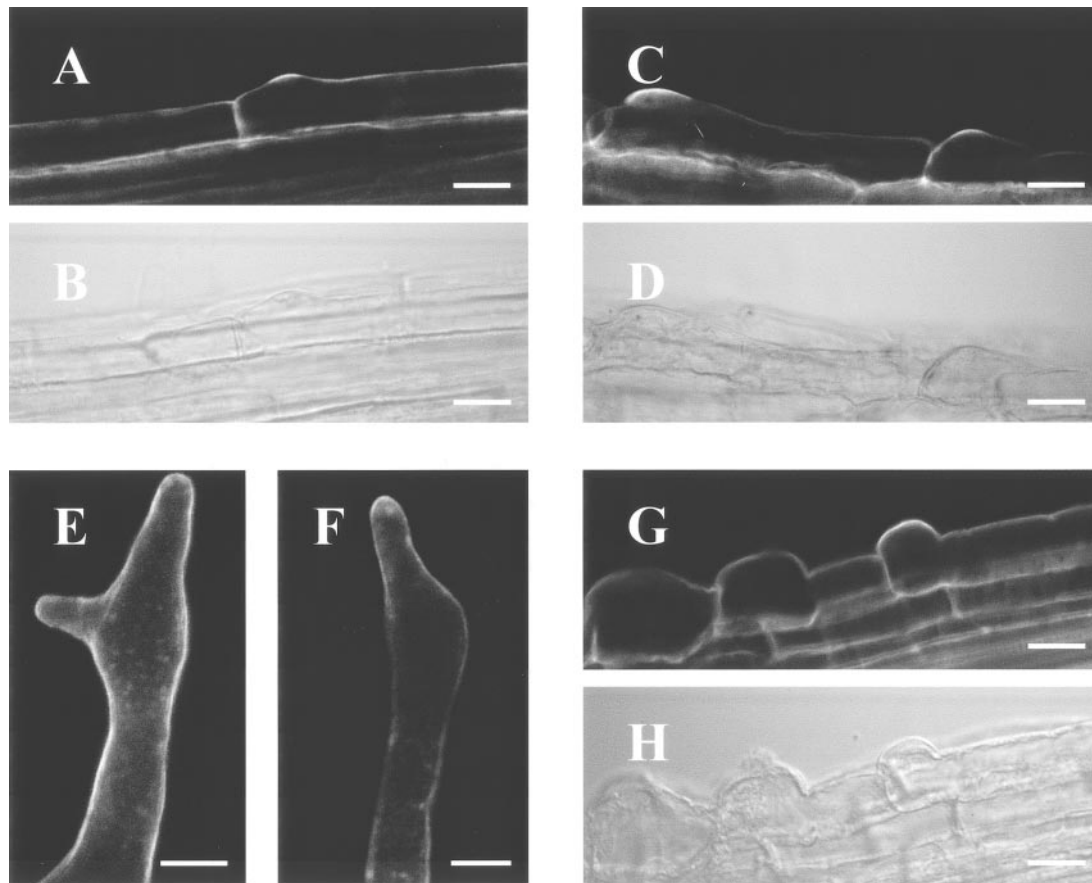
was challenged by looking at the localization of XET action in roots of different mutants, affected on different sites of the root hair formation pathway.

The *root hairless1* (*rhl1*) mutant completely lacks root hair initiation (Fig. 6A). On *rhl1* roots, no local fluorescent patches could be detected at higher magnification (Fig. 6B). This was also the case on roots of *rhd6-1* (data not shown).

The other mutants tested were not affected in root hair initiation but were affected in the later stadia of root hair growth. In all mutants, a local increase of XET action also accompanied root hair initiation when the trichoblasts or the root hairs had aberrant forms or sizes. It also turned out that in all mutants that developed root hairs, the wall of the root hairs exhibited a uniform signal of XET action, irrespective of the shape, the size, or the fate of the root hair.

The *rhd1* mutant produces root hairs that are comparable with wild-type root hairs. The distinctive

feature, however, is the bulbous region at the base of the hair. A site with high XET action was present on the swollen trichoblasts at the site where the root hair was going to emerge in a later stadium (Fig. 6C, see arrow). An older root hair with a clear bulbous region showed high XET action in the whole cell wall (Fig. 6D). In *rhd2*, a mutant that is characterized by its root hairs' failure to elongate, high XET action marks the initiation site (Fig. 6E). A root hair that had ceased elongation (Fig. 6F) displayed high XET action throughout the wall. Comparable data on XET action were found in mutants that carry mutated genes that are needed sequential to *RHD2*. In *shv1-4* root hairs behaved as in *rhd2*, they exhibited high XET action as well in young (Fig. 6G) as in older stadia (Fig. 6H) but failed to become long and finger shaped like normal wild-type root hairs. In *shv2-1*, the initiation site was marked by high XET action (Fig. 6I). Young root hairs displayed a XET action pattern reminiscent



**Figure 4.** Fluorescent XET action donor substrate colocalization during initiation and subsequent growth of root hairs in wild-type *Arabidopsis* treated with cytoskeleton and cellulose synthesis inhibitors. A and B, High XET action confined to the forming bulge in a root treated with latrunculin. B. Fluorescence (A) and bright-field image (B). C and D, Two swollen trichoblasts after oryzalin treatment still showing a localized increase of XET action. Fluorescence (C) and bright-field image (D). E and F, Two branching root hairs caused by oryzalin treatment show homogeneous XET action over the entire surface. G and H, Root after 2,6-dichlorobenzonitrile (DCB) treatment shows swollen cells but root hairs initiate at the expected site. High XET action is localized to the bulge. Fluorescence (G) and bright-field image (H). Bars = 20  $\mu\text{m}$ .

of that found in wild-type roots (Fig. 6J). Further up the root, where short (Fig. 6K) as well as long (Fig. 6L) root hairs occurred, they all showed high XET action throughout the wall. In *shv3-1*, the number of longer root hairs was increased compared with *shv2-1*, but the action pattern of XET was the same (data not shown). In *cow1-2*, root hairs failed to grow beyond a certain length. In this mutation, high XET action was present at initiation (Fig. 6M) and in older root hairs (Fig. 6N) that failed to elongate further.

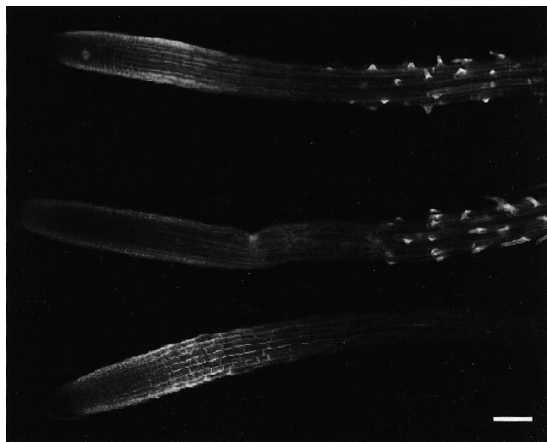
## DISCUSSION

When entering the differentiation zone, the elongating root cells that are programmed to become trichoblasts drastically add a new growth pattern to allow the highly localized emergence of root hairs. The initiation of a root hair is characterized on the level of gene expression patterns (for review, see Schiefelbein, 2000). On the level of cell physiology, specific enzymes or proteins need to restructure a defined spot of the apical outer periclinal cell wall to

allow local wall loosening and bulging. At the time of root hair initiation, inside the cytoplasm actin and microtubules rearrange (Emons and Derksen, 1986; Baluška et al., 2000a, 2000b). A highly localized acidification (pH 4.5) of the cell wall is associated with the initiation process (Bibikova et al., 1998). Once the initiation is completed, the wall pH returns to the pH (approximately 6) found in the rest of the trichoblast. Besides pH changes, other factors are likely to be important to predict the future site of root hair emergence because artificial acidification of the entire trichoblast wall did not alter this site.

Expansins, which are cell wall-loosening enzymes (Cosgrove, 2000), were detected in the outgrowing bulges (Baluška et al., 2000a), whereas their action could not be assayed. However, it is not clear if expansin is also involved in the initiation of the root hair, i.e. the definition of the site and the preparation of the wall before bulge formation.

Because developmentally regulated changes in cell wall structure may be required for normal growth



**Figure 5.** Colocalization of XET action and its fluorescent donor substrate during the initiation and subsequent growth of a root hair in wild-type *Arabidopsis* roots kept at different pH during 6 h. The upper root at pH 5.5 displays normal XET action in the elongation zone and root hairs. The middle root at pH 4.5 shows a reduction of the action specifically in the elongation zone. The lower root at pH 7.0 completely lacks root hair initiation, but has a high action in the elongation zone. Bar = 100  $\mu\text{m}$ .

and highly specialized differentiation (e.g. Belanger and Quatrano, 2000), we investigated when and where XET acts during the initiation and subsequent tip growth of root hairs. Using a fluorescence technique to visualize where endogenous XET acts on endogenous xyloglucan (as donor substrate), we detected a highly localized up-regulation of XET action in trichoblasts precisely and only at the site of future root hair formation, before any visible bulging occurred, and later in the wall of the emerging root hair. Because the trichoblasts, which are situated at the end of the elongation zone, mainly possess transverse arrays of cellulose microfibrils (Verbelen and Kerstens, 2000), highly localized concerted action of expansin and XET could be necessary to loosen the wall. Turgor pressure-driven separation of adjacent microfibrils and the possibility to deposit new cell wall material between these loosened microfibrils could lead to bulge formation. Tip growth subsequently is initiated in the bulge, giving rise to a young root hair. In root hairs, the level of XET action was uniform throughout the whole cell wall.

The highly localized up-regulation of XET action is inextricably bound up with root hair initiation. We never found root hair initiation without high XET action or a patch of high XET action without root hair initiation. Therefore, the patch of high XET action was present in trichoblasts of all mutants in root hair growth we tested. It was absent in specific root hair initiation mutants (*rhl1*, *rhd6-1*, and parts of *axr2-1*) and in wild-type roots where root hair initiation is inhibited by high pH (7.0) or AVG (ethylene synthesis inhibitor).

As a consequence, the localization of high XET action is controlled at the hormone level in parallel

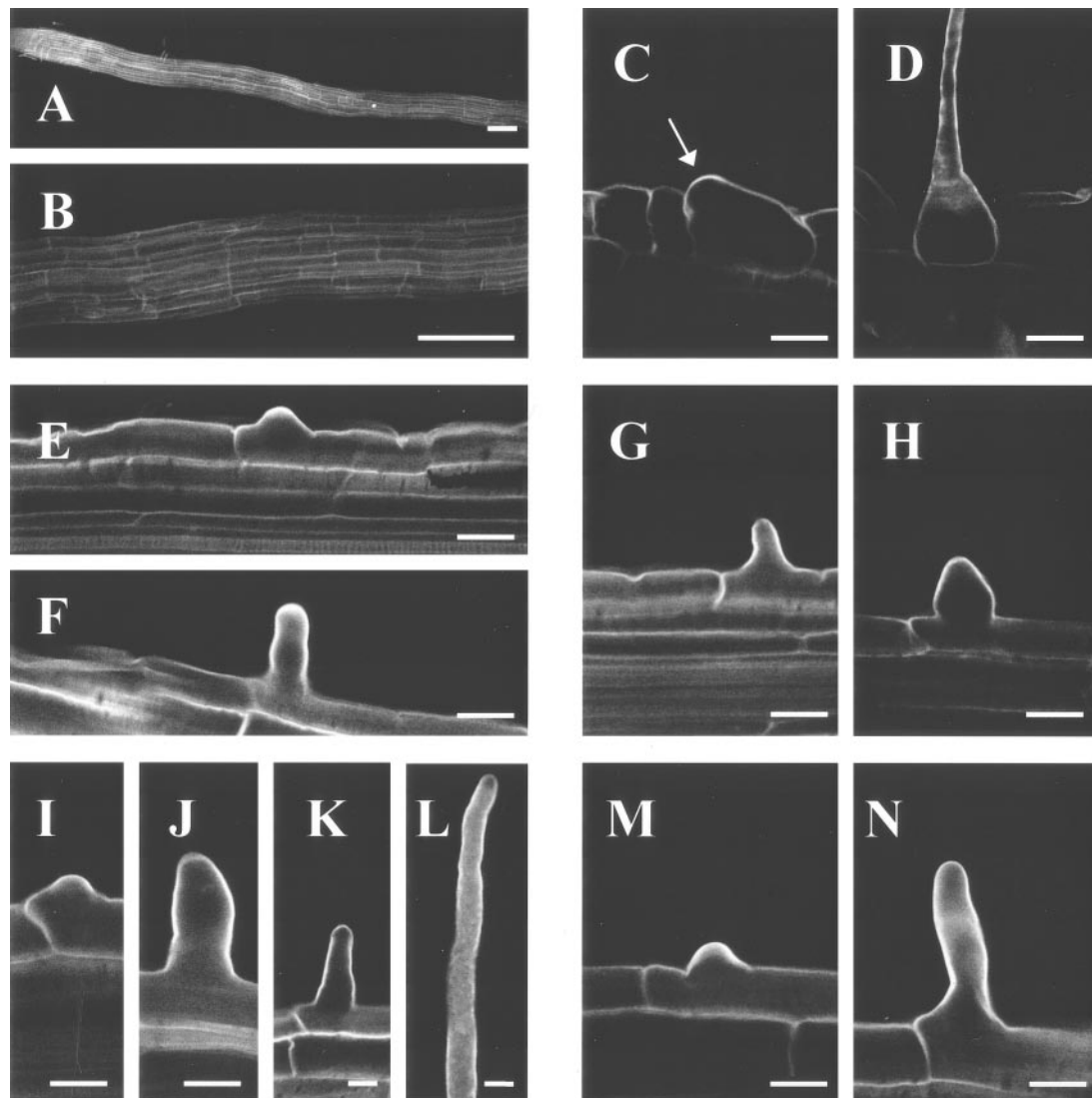
with the initiation of new root hairs. Both auxin and ethylene are positive regulators of root hair development in *Arabidopsis* after the *TTG/GL2* pathway has established the early differentiation events (Masucci and Schiefelbein, 1994, 1996; Tanimoto et al., 1995; Pitts et al., 1998). In this study, we have shown that ethylene plays a role in determining the pattern of XET action. Application of ACC, mimicking the *ctr1-1* mutant, led to the initiation of root hairs closer to the root tip and this is accompanied by high XET action at the sites of root hair outgrowth in very short trichoblasts. AVG, an ethylene biosynthesis inhibitor, led to the loss of root hair formation and completely abolished the localized increase in XET action. In the ethylene-resistant mutant *etr1-3*, both root hair initiation and localized XET action occur on more proximal parts of the root compared with wild-type roots.

As would be expected (Tanimoto et al., 1995; Masucci and Schiefelbein, 1996; Pitts et al., 1998), auxin did not induce ectopic root hair formation, neither did it change the pattern of sites with high XET action in the roots. However, the auxin resistant mutant *axr2-1* forms fewer root hairs. Concomitantly, fewer patches of high XET action were detected on the potential trichoblasts.

The localization of high XET action at the initiation site is not sensitive to cytoskeleton inhibitors. Root hair growth on the contrary is sensitive to the inhibitors. In the presence of F actin-disrupting drugs, the high XET action was present and root hair bulges were formed. Subsequent outgrowth of the newly formed hair failed, however. It is known that bulge formation involves mechanisms different from tip growth (Ridge, 1995; Miller et al., 1999). Turgor-mediated bulging of a cell wall loosened by XET and other synergistically acting wall proteins such as expansins or endoglucanases (Fry, 1994; Catalá et al., 1997; Baluška et al., 2000a; Cosgrove, 2000) can probably still occur without F actin being present. Interference with actin, on the other hand, has its consequences on vesicle targeting and delivery at the growing tip of the root hairs (Miller et al., 1999). Also, microtubule antagonists did not affect the localized up-regulation of XET action and the subsequent bulge formation. Root hair growth was affected. Root hairs bifurcated due to the formation of additional growth points. The initiation of a new growing tip occurred without any local up-regulation of XET action. In this respect, the onset of tip growth thus is clearly different from the initiation of the root hair itself. Our data confirm earlier findings that the only effects of microtubule antagonists in growing root hairs are the loss of growth directionality and the formation of multiple growth points (Bibikova et al., 1999).

In roots treated with DCB, the typical patches of high XET action occurred in cells that had already acquired an elongated form. Cells affected in an earlier stage of development completely lost the ability





**Figure 6.** Colocalization of XET action and its fluorescent donor substrate in specific root hair mutants of Arabidopsis. A, The root hairless1 (*rh1*) mutant shows high fluorescence in the elongation zone and a complete lack of root hair initiation and of the concomitant XET-caused fluorescence. B, Detailed picture of the XET assay in *rh1* showing the complete lack of local fluorescent patches. C and D, XET action in *rh1*. The arrow points to a region with high XET action in a typically swollen trichoblast where the future root hair will grow out (C). In a later stage, the growing root hair shows high XET action all over its surface (D). E and F, XET action in *rh2*. XET action is high in root hair bulges (E). Further up the root, root hairs that fail to elongate display XET action similar to normal wild-type root hairs (F). G and H, XET action in *shv1-4*. XET action is high in young (G) and older root hairs that fail to grow further (H). I through L, XET action in *shv2-1*. In young parts of the root XET action is high in bulges (I) and throughout elongating root hairs (J). In older parts of the root, short root hairs are found among long root hairs exhibiting the same high XET action (K and L). M and N, XET action in *cow1-2*. The *cow1-2*-mutant produces short root hairs that fail to elongate as wild-type root hairs. XET action is nevertheless high in bulges (M), whereas the whole root hair cell wall shows normal XET action (N). Bars = 20  $\mu\text{m}$ , except in A and D, where bars = 100  $\mu\text{m}$ .

for anisotropic growth and for initiation of root hairs. In these cells, no sites with high XET-action could be detected consistently.

High XET action thus occurs in the elongation zone (Vissenberg et al., 2000), at sites of root hair initiation, and in the wall of root hairs. Therefore, different isoforms of XET could be active at specific locations in the root. In the first case, XET action is spread throughout expanding walls but is not the only de-

termining factor (enzyme) for expansion. In the second case, it is limited to a spot of wall bulging. In the third case, it is present in walls that do not expand anymore.

Known XETs have pH optima of about 5.0 to 6.5 (Steele and Fry, 2000), a range typical for the apoplast environment but clearly different from the pH (approximate pH 4.5) at the site of root hair initiation (Bibikova et al., 1998). At pH 4.5, we still found high

XET action in initiating root hairs, but not any longer in the elongation zone of the root. At pH 7.0 the situation is inverted; the elongation zone exhibits high action, but root hair initiation is completely inhibited as is the concomitant XET action. This indicates that an unusually acidophilic isoform of XET is present before and during bulge formation that has a specific role in root hair initiation.

The XET action occurring throughout the wall of the growing root hair has other characteristics. It was detected throughout the pH range tested. It was highest when assayed at pH 5.5, and lower at pH 7.0 and pH 4.5. This XET action also differs from that linked to root hair initiation by its temporally and spatially uniform distribution, as exemplified during the formation of new growing tips during oryzalin treatment. Because root hair growth is limited to the tip, this type of XET action therefore seems rather to be linked to wall deposition, but not expansion, thus strengthening these walls. The potential roles of different XET isoforms in root and root hair growth are summarized and visualized in Table I and Figure 7.

To conclude, we can state that root hair initiation is accompanied by a highly localized increase of XET action that is specific in its pH dependence and insensitive to disturbance of the cytoskeleton. We propose that local wall loosening established by XET and expansins (possibly in cooperation with other cell wall proteins such as arabinogalactan proteins; Samaj et al., 1999) is necessary for root hair formation.

## MATERIALS AND METHODS

Plants of *Arabidopsis* wild type (ecotypes Columbia-0 and Wassilewskija) and mutants (*etr1-3*, *ctr1-1*, *axr2-1*, *rhl1*, *rhd1*, *rhd2*, *rhd6-1*, *shv1-4*, *shv2-1*, *shv3-1*, and *cow1-2*) were vertically grown from seed under sterile conditions on a Murashige and Skoog medium without hormones (4.7 g L<sup>-1</sup>; Duchefa, Haarlem, The Netherlands), supplemented with 10 g L<sup>-1</sup> Suc. The culture medium was adjusted to pH 5.5 and solidified with 4 g L<sup>-1</sup> Gelrite (Duchefa). Healthy roots were obtained after 4 to 5 d and used for further experiments.



**Figure 7.** Schematic representation of the distribution of XET isoforms in the epidermis of a growing *Arabidopsis* root.

Inhibitors of F actin, microtubules and cellulose deposition, latrunculin B (Calbiochem, La Jolla, CA), oryzalin (Alltech Associates, Laarne, Belgium), and DCB (Fluka Chemika, Buchs, Switzerland) were made as stock solutions (all in dimethyl sulfoxide) and added to the culture medium at a final concentration of 1.25, 10, and 10  $\mu$ M, respectively. 1-Aminocyclopropane-1-carboxylic acid (ACC; Sigma, St. Louis), a precursor of the gaseous plant hormone ethylene, AVG (Sigma) an ethylene biosynthesis inhibitor and the auxin IAA (Sigma) were used at a concentration of 5, 10, and 5  $\mu$ M, respectively. Roots were incubated for 4 to 6 h on the Murashige and Skoog medium supplemented with the various inhibitors, ACC, or IAA before the assay itself. To study the effect of different pHs on root hair initiation, roots were incubated for 6 h in 25 mM MES at pH 4.5, 5.5, and 7.0, respectively, before XET assaying. Effectiveness of the inhibitors, the ethylene precursor, the IAA, and the different pHs were controlled before the cytochemical XET assays.

Xyloglucan-endotransglycosylase (XET) action was demonstrated as described by Vissenberg et al. (2000). In brief, roots were incubated in a 6.5  $\mu$ M XGO-SR mixture (XLLG-SR > XXLG-SR > XXXG-SR; for nomenclature, see Fry et al., 1993; for the synthesis, see Fry, 1997) dissolved in Murashige and Skoog culture medium at pH 5.5 (or as indicated in "Results") for 1 h. The assay was followed by a 10-min wash in ethanol:formic acid:water (15:1:4, v/v/v) and an incubation overnight in 5% (w/v) formic acid. Cellotetraose-SR and cellobiose-SR-solutions were used also at a concentration of 6.5  $\mu$ M as controls for XET assaying followed by the same two washes as described above.

**Table I.** Properties of three putative XET isoforms, active during root growth and differentiation in *Arabidopsis*

Color on Diagram	Black	Grey	Dotted
Location	Elongation zone, cortex + epidermis	Bulge	Side walls of root hair
Subcellular Distribution	Uniform over area of cell wall	Highly localized (in discrete patches)	Uniform over area of cell wall
Action at pH 4.5	Low	High	Lower
Action at pH 5.5	High	High	High
Action at pH 7.0	High	Absent; no new bulges formed at pH 7	Lower
Proposed role	Root elongation	Bulge initiation	Side wall strengthening, incorporation of newly deposited material

Fluorescent and bright-field pictures were made using the 514-nm laser line of a MRC 600 confocal laser scanning microscope (Bio-Rad, Hercules, CA) mounted on a Axioskop (Zeiss, Jena, Germany) and equipped with a 40× water immersion objective (numeric aperture = 0.9) and a 10× objective (numeric aperture = 0.3).

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