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Expression of α-expansin genes during root acclimations to O_2 deficiency in *Rumex palustris*

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Abstract

Thirteen α-expansin genes were isolated from *Rumex palustris*, adding to the six already documented for this species. Five α-expansin genes were selected for expression studies in various organs/tissues of *R. palustris*, with a focus on roots exposed to aerated or O_2-deficient conditions, using real-time RT-PCR. Several cases of differential expression of α-expansin genes in the various root types of *R. palustris* were documented, and the identity of the dominant transcript differed between root types (i.e., tap root vs. lateral roots vs. adventitious roots). Several genes were expressed differentially in response to low O_2. *In situ* hybridizations showed expansin mRNA expression in the oldest region of the tap root was localized to cells near the vascular cambium; this being the first report of expansin expression associated with secondary growth in roots. *In situ* hybridization also showed abundant expression of expansin mRNA in the most apical 1 mm of adventitious roots. Such early expression of expansin mRNA in cells soon after being produced by the root apex presumably enables cell wall loosening in the elongation zone of roots. In addition, expression of some expansin mRNAs increased in 'mature zones' of roots; these expansins might be involved in root hair formation or in formation of lateral root primordia. The present findings support the notion that large gene families of α-expansins enable flexibility in expression for various organs and tissues as a normal part of plant development, as well as in response to abiotic stress.

Introduction

Expansins are proteins that mediate cell wall loosening (McQueen-Mason *et al.*, 1992; McQueen-Mason and Cosgrove, 1995), a process essential for cell elongation, organ morphogenesis and cell differentiation (Cosgrove, 2000a, b). Two main types of expansins have been described, α- and β-expansins; each being a large gene family in the species studied to date (tobacco, Link and Cosgrove, 1998; tomato, Catalá *et al.*, 2000; arabidopsis and rice, Lee *et al.*, 2001; maize, Wu *et al.*, 2001a). The available data indicate α-expansins have greatest 'loosening activity' towards walls of dicots, whereas the β-expansins are most active on walls of monocots (Cosgrove, 2000a; Lee *et al.*, 2001). The large gene families are hypothesized to enable temporal and spatial regulation of organ-, tissue-, and cell-specific expression of expansins during plant development, differentiation, and in

responses to abiotic stresses (Lee *et al*., 2001). Although the majority of studies have focused on the role of expansins in cell (and organ) elongation (Cosgrove, 2000a), expansins have also been implicated in developmental processes (e.g. fruit ripening, Rose *et al*., 2000) and in regulation of organ morphogenesis (e.g. leaves, Fleming *et al*., 1997; Pien *et al*., 2001).

The semi-aquatic species *Rumex palustris* has been used as a dicot model system to study the hormonal and molecular basis of submergence-induced hyponastic growth and petiole elongation (Peeters *et al*., 2002; Cox *et al*., 2003; Voesenek *et al*., 2003). Petiole elongation upon submergence results from cell elongation only (Voesenek *et al*., 1990); a process corresponding with the up-regulation of α-expansins (Vriezen *et al*., 2000; Vreeburg, 2004). Similarly, stem elongation in submerged deepwater rice involves organ-specific expression of expansins (Kende *et al*., 1998; Lee and Kende, 2001). Submergence-induced elongation enables the shoot to remain in, or re-gain, contact with air, providing a 'snorkel' for entry of O_2 and escape of CO_2 and ethylene (Jackson and Armstrong, 1999; Voesenek *et al*., 2004). Semi-aquatic plants typically contain large volumes of aerenchyma (interconnected gas-filled spaces) that provide a low-resistance internal pathway for gas transport from shoot to root extremities (Armstrong, 1979; Colmer, 2003).

O_2 deficiency is a feature of flooded soils (Ponnamperuma, 1984), and *R. palustris* produces numerous adventitious roots in response to these conditions (Visser *et al*., 1996a), a common adaptation among wetland species (Armstrong, 1979; Jackson and Drew, 1984; Vartapetian and Jackson, 1997). The adventitious roots of *R. palustris* contain extensive 'honeycomb-type' schizogenous aerenchyma (Visser *et al*., 2000). This type of aerenchyma develops in the cortex by cells being forced apart due to oblique divisions by some of the cells in radial rows, with the numbers of divisions, and therefore gas-space volume in the roots, depending upon the species (Justin and Armstrong, 1987). Aerenchyma also forms in the tap root in *Rumex* species from wet habitats (Laan *et al*., 1989). This aerenchyma would enhance O_2 supply to the tap root, as well as enabling O_2 diffusion to primary lateral roots and newly formed secondary lateral roots that emerge from this organ (see Laan *et al*., 1989).

Expansins might, in addition to regulation of petiole elongation during submergence (Vriezen *et al*., 2000; Vreeburg, 2004), play a role in acclimation of root systems to soil flooding. Expansins have been implicated in the initiation of adventitious roots (e.g. from the hypocotyl of loblolly pine; Hutchison *et al*., 1999), and high levels of expansin mRNA were observed in adventitious root primordia within stem nodes of deepwater rice (Cho and Kende, 1998). A role for expansins in formation of aerenchyma, a process that typically involves cell-wall separations, has been hypothesized by Jackson and Armstrong (1999), but not yet studied.

The experiments described in the present paper: (i) Extend our earlier work to isolate expansin genes from *R. palustris* (Vriezen *et al*., 2000), with 13 new α-expansins. (ii) Show, using real-time RT-PCR, specific expression patterns of some α-expansin genes in different root types, and that mRNA abundance is influenced by O_2 deficiency. (iii) Localize α-expansin mRNA in root tissues, using *in situ* hybridizations, showing highest expansin expression in growing tissues (i.e., tips of adventitious roots and near the vascular cambium in tap roots). Expression was always analyzed for plants grown in aerated or in stagnant nutrient solutions. This latter treatment mimics the changes in root-zone gas composition (i.e., O_2 deficiency with increased ethylene and CO_2) as occurs during soil waterlogging (Wiengweera *et al*., 1997), and strongly induces adventitious rooting in *R. palustris* (Visser *et al*., 1996b).

Results

Isolation of α-expansin genes from R. palustris

Earlier work by our group isolated 6 α-expansin genes (1 cDNA and 5 genomic fragments) using degenerated primers based upon sequences in GenBank (Vriezen *et al*., 2000). *RpEXPA1* (acc. # AF167360, gene nomenclature according to Kende *et al*., 2004) was a full-length cDNA isolated from a library made using leaf RNA of *R. palustris* exposed to 24 h of submergence (Vriezen *et al*., 2000). In the present work, *RpEXPA1* was used as a probe to screen a cDNA library made using RNA from the uppermost 5–7 mm of tap root of plants after 48 h in stagnant de-oxygenated nutrient solution. Nine cDNAs were isolated, 7 of

which were full length (*RpEXPA7* to *RpEXPA14*, acc. #'s AF428174-181) and one partial length (*RpEXPA18*, acc. # AF428185). In addition, the degenerated primers described by Vriezen *et al.* (2000) were used to isolate cDNAs from RNA extracted from the uppermost 5–7 mm of tap root, 5 mm tips of adventitious roots, and petioles; sampled 10 d after growth in de-oxygenated stagnant nutrient solution. These experiments resulted in isolation of the partial sequences *RpEXPA16* and *RpEXPA17* (acc. #'s AF428183 and AF428184, respectively) from the upper tap root, *RpEXPA15* (acc. # AF428182) from adventitious root tips, and *RpEXPA19* (acc. # AF428186) from petioles. In summary, 13 α-expansin genes were isolated, in addition to the six described by Vriezen *et al.* (2000).

Amino acid sequence alignment of the 19 α-expansins from *R. palustris*, showed that these genes are highly homologous (Figure 1). Similar findings are reported for α-expansin gene families in other species (Catalá *et al.*, 2000; Wu *et al.*, 2001a; Lee *et al.*, 2001). The similarity amongst the α-expansins in *R. palustris* ranges from 63.3% (*RpEXPA10* and *RpEXPA11* compared with *RpEXPA18*) to 99.6% (*RpEXPA13* compared with *RpEXPA14*). When the signal peptide sequence is not included, the similarity is even higher, being 66% (*RpEXPA2* compared with *RpEXPA12*) to 100% (*RpEXPA13* compared with *RpEXPA14*). The similarity between the α-expansins in *R. palustris* and those in other plant species ranges from a lowest value of 43.3% (*RpEXPA18* compared with *LeEXPA3* from tomato, acc. # AF059487) to a highest similarity of 89.0% (*RpEXPA4* compared with *CsEXPA1* from cucumber, acc. # U30382) (data not shown).

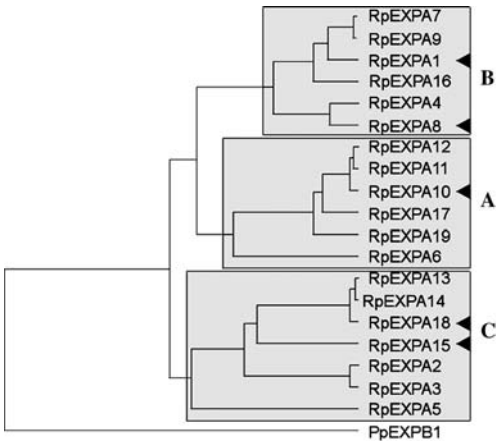

Figure 1. Phylogenetic tree showing the similarities of the deduced amino acid sequences for 19 α-expansins isolated from *Rumex palustris*. The tree was constructed based on predicted amino acid sequences of protein regions using DNAstar software as described in the section Materials and Methods, with the β-expansin from *Phleum pratense* (*PpEXPB1*, acc. # U78813) as out group. The GenBank accession numbers of the *R. palustris* expansins are given in the text in the section 'Results'. The genes grouped into 3 main clades. The five genes studied by real time RT-PCR are indicated (see text for details).

The phylogenetic analysis showed that the *R. palustris* α-expansins grouped into three main clades (Figure 1). Groups within α-expansin gene families were also identified in analyses of gene sequences isolated from other species (Link and Cosgrove, 1998; Catalá *et al.*, 2000). Comparison of the present sequence data on α-expansins in *R. palustris*, with those published (Link and Cosgrove, 1998) showed the clade of genes in *R. palustris* closest to the 'out group' of our analysis (*Phleum pratense* β-expansin 1, *PpEXPB1*, acc. # U78813) was most similar to the 'C group' (groups as defined in Link and Cosgrove, 1998), the next closest clade was most similar to the 'A group', while the most divergent clade had similarities to the 'B group'.

We selected five α-expansins for studies, using real-time RT-PCR, to evaluate abundances of mRNAs in a range of organs/tissues of plants either grown in aerated or exposed to stagnant solutions. The five genes selected were based on (i) having representatives from each of the three clades, and (ii) the ability to make gene-specific primers/probe combinations. The five α-expansin genes studied are shown by arrows in Figure 1 and are listed in Table 1.

Adventitious rooting and schizogenous aerenchyma are enhanced by growth in stagnant solution

The effects of stagnant conditions on the formation of adventitious roots, and porosity in adventitious, tap, and primary lateral roots, were assessed. Upon transfer to stagnant conditions, new roots emerged from, or above, the root-shoot junction and also from the upper region of the tap root (Figure 2A and 2B). Laan *et al.* (1989) termed these 'adventitious' and 'secondary lateral' roots, respectively. Roots from these two origins were morphologically indistinguishable. Overall, these newly formed roots are white and thicker

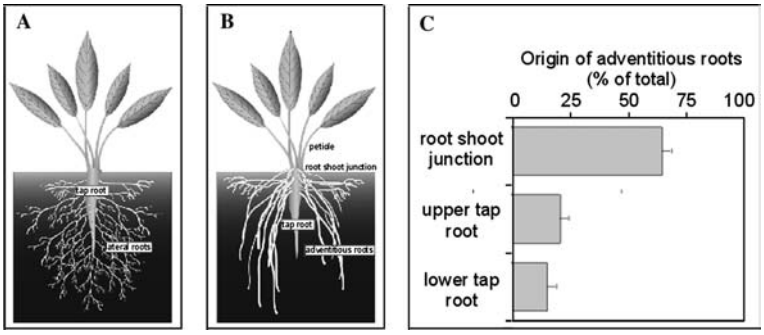

Figure 2. Drawing of *Rumex palustris* showing the main root types of plants grown in (A) aerated or (B) de-oxygenated stagnant nutrient solution. (C) shows the regions and numbers of adventitious roots formed by plants either in continuously aerated or stagnant de-oxygenated nutrient solution for the final 10 d. Upper tap root = top 1 mm. Data given are means ± standard errors ($n = 3$).

Table 1. Sequences of Taqman probes and primers combinations for the five expansin genes studied using real time RT-PCR.

Gene	Probe 5′-*6-FAM….TAMRA*-3′	Forward primer 5′-…-3′	Reverse primer 5′-…-3′
RpEXPA1	ggacatcgcctgccagccg	agacgttcactcggtgtcgat	cagttctggccccaattcc
RpEXPA8	ctgtcattctccgttacctctagcgacgga	caactcttaccttaacggccaaac	ccccaacgtaagtctgaccaaa
RpEXPA10	cgccgcagctcatcccgttg	ttgtggattcggcaacttgtact	tgggtcgtcagcgcactt
RpEXPA15	tcgcccaccctgtcttccagca	ccctcccctgcaccactt	agggatgatgccggcttt
RpEXPA18	ccgtgaccacaatgctgccgg	tgtgtggctgaccggaaat	tgttcggcgggcagaa
Rp18S	acctcgacggatgccacggcc	ccgttgctctgatgattcatga	gttgatagggcagaaatttgaatgat

Table 2. Porosity of tap, lateral, and adventitious roots of *Rumex palustris* grown in either continuously aerated or in stagnant de-oxygenated nutrient solution for the final 10 d. The apical 120 mm of lateral and adventitious root tissues were used. Data given are means ± standard errors ($n = 4$).

Tissue	Treatment	
	Aerated	Stagnant
	Porosity (% gas spaces/volume)	
Tap root	6.2 ± 0.23	13.0 ± 0.41
Lateral roots	20.6 ± 1.90	23.8 ± 3.13
Adventitious roots	26.8 ± 1.80	38.4 ± 0.83

than the pinkish primary lateral roots (see also Laan *et al.*, 1989, for other species of *Rumex*). For simplicity, hereafter we refer to all these newly formed roots as 'adventitious roots'.

In response to the stagnant O_2 deficient treatment, *R. palustris* produced 49 ± 4 adventitious roots during 10 d; with the majority of these emerging from the root/shoot junction (Figure 2C). Plants continuously growing in aerated solution formed only 11 ± 2 of these roots.

Porosity in adventitious roots that grew in aerated solution was 27%, and increased to 38% for plants in stagnant solution (Table 2). This development of gas spaces was enhanced along the entire length of adventitious roots, but the relative increase in porosity was greatest towards the apex (Figure 3A). The higher porosity in adventitious roots grown in stagnant solution was associated with these roots having a greater diameter (Figure 3B). Root diameter had reached its maximum value by 10 mm behind the tip of aerated adventitious roots, but in stagnant conditions the diameter continued to increase, although gradually, up to 30 mm behind the tip (Figure 3B). The increased porosity (and diameter) of roots grown in stagnant solution resulted from a greater number of cell divisions within the cortex to produce larger gas-filled voids. For example, at 5 mm behind the root tip the largest gas-filled voids in aerated roots were typically boarded by 10 ± 1 cells, whereas in stagnant solution there were 17 ± 0.7 cells ($n = 5$). This response of increased

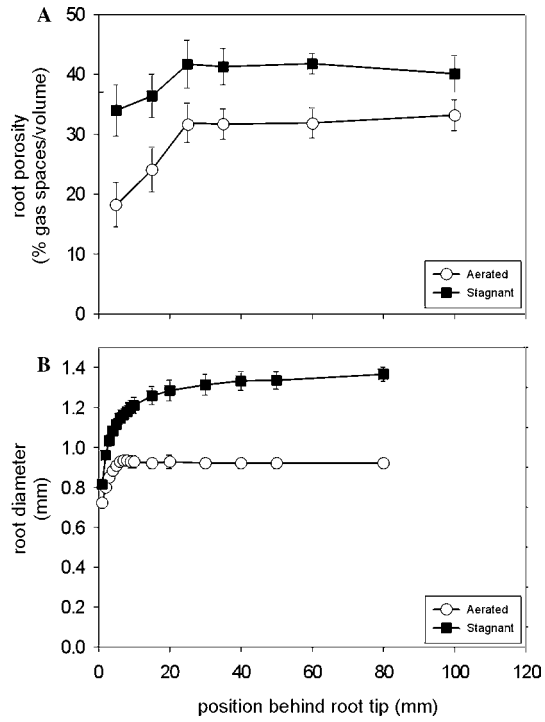

Figure 3. Distributions of (A) porosity and (B) diameter along adventitious roots of *Rumex palustris* grown either in aerated or in de-oxygenated stagnant nutrient solution for the final 10 d (imposed on 5 week-old plants). Data given are means ± standard errors ($n = 3$). Aerated plants in (B) were grown continuously in aerated solution, and in (A) were given a short period in stagnant solution to induce adventitious roots, and plants were then transferred back to aerated solution into which the roots emerged and grew (performed to obtain sufficient roots for sampling).

cell divisions was evident for all the gas-filled voids within the root cortex. The average size of cortical cells (at 5 mm behind the tip) did not differ between roots from the two treatments (data not shown).

Figure 4 shows a series of transverse sections taken at increasing distances behind the apex of adventitious roots grown in aerated or stagnant O_2 deficient solution. The largest gas-filled spaces were located in the mid-cortex, whereas the more basal sections in Figure 4 show only the outer-cortex, so the numbers of cells surrounding each void do not match the values given in the preceding paragraph. The series of sections show that growth in stagnant solution enhances formation of schizogenous aerenchyma within 250 μm of the apex. Numerous examples of cells in radial rows that have undergone recent divisions can be seen in

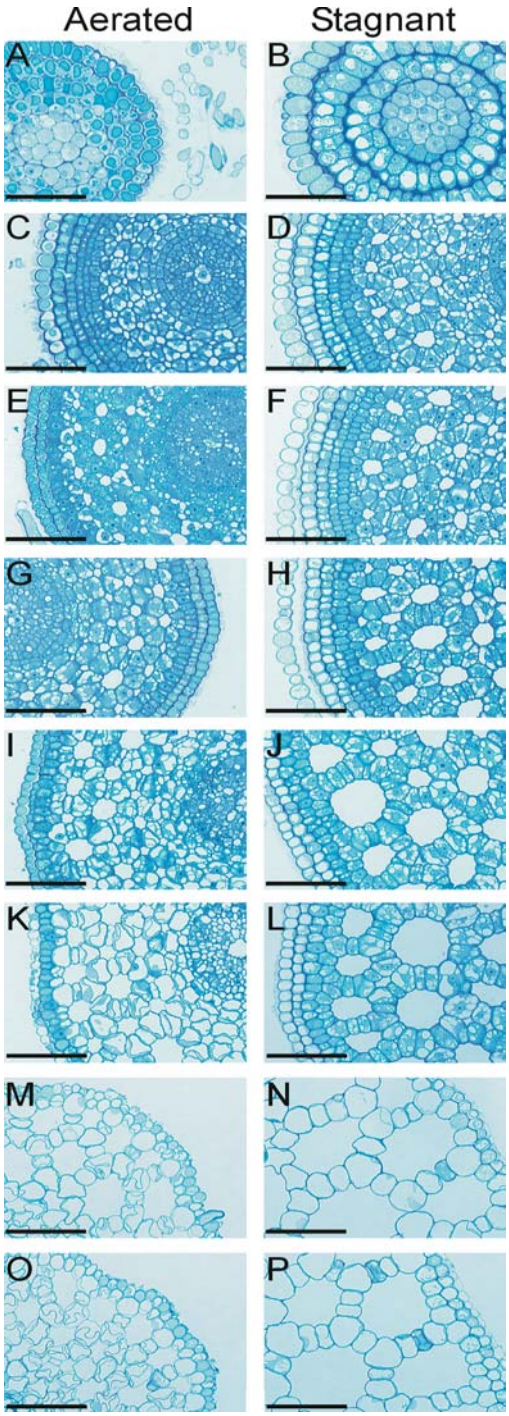

Figure 4. Series of transverse sections taken from various distances (A, B/50 μm: C, D/250 μm: E, F/500 μm: G,H/750 μm: I, J/1 mm: K, L/1.5 mm: M, N/5 mm and O, P/9 mm respectively) behind the tip of adventitious roots of *Rumex palustris* either grown in aerated or in stagnant O_2 deficient nutrient solution. Treatments are given in the Figure. Bars represent 100 μm.

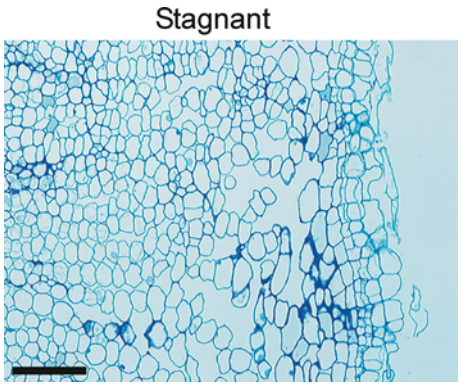

Figure 5. Transverse section taken from the upper 3 mm of the tap root of *Rumex palustris* after 10 d exposure to stagnant O_2 deficient nutrient solution. Bar represents 100 μm.

the various sections. The series of sections also show that enhanced formation of the honeycomb-type schizogenous aerenchyma induced by the stagnant treatment results from promotion of the number of divisions by cells in the radial rows, and not due to differential cell expansion.

Tap roots of plants exposed to stagnant solution for 10 d doubled in porosity (Table 2), due to formation of aerenchyma in the secondary cortex (Figure 5). The aerenchyma in the tap root is also schizogenous, but not of a highly organized honeycomb-type as in the adventitious roots. Aerenchyma in the tap root would be vital for O_2 diffusion to adventitious roots that emerge from this organ, as well as to the primary lateral roots. Primary lateral roots grown in aerated solution had a porosity of 21%, and this hardly changed after 10 d in stagnant solution (Table 2).

In summary, the stagnant treatment resulted in (i) enhanced aerenchyma development in the secondary cortex of the tap root, (ii) initiation of adventitious rooting from the shoot base and upper tap root, and (iii) adventitious roots with greater diameter and gas-filled porosity, when compared with those of plants in aerated solution. The expression of expansins in these various root tissues was examined, and described below.

α-expansin genes are expressed differentially between and within root types, and are influenced by O_2 deficiency

The existence of variation in root types, and different tissues within these, as well as the high plasticity shown by the root system of *R. palustris* in response to root-zone O_2 deficiency, makes this a suitable system to evaluate the transcript profiles of the expansin multi-gene familiy.

The relative mRNA abundances of the five α-expansin genes studied (Figure 1 and Table 1) in various tissues of *R. palustris* are shown in Figure 6. For each expansin tested, mRNA abundance in the various samples is expressed relative to that in the tissue with the highest transcript level. The data show large variation in the expression of a particular expansin across tissues and between the two root-zone O_2 treatments. *RpEXPA1* was expressed most abundantly in petioles compared with the other tissues, and root-zone O_2 treatment only had a minor effect. *RpEXPA8* expression in aerated plants was most abundant in mature zones of lateral roots, and low O_2 treatment caused expression of this gene to decrease in that tissue. By contrast, *RpEXPA8* mRNA abundance increased in all other tissues when roots were exposed to stagnant conditions. *RpEXPA10* and *RpEXPA18* were hardly expressed in lateral roots, but were both expressed in the main tap root, and in the latter organ expression of both genes increased in stagnant conditions. *RpEXPA15* was most abundant in lateral root tips, and expression in these tips increased in stagnant conditions.

Spatial patterns of expression of the five α-expansin genes along adventitious roots were assessed (Figure 7). The relative abundance of *RpEXPA1* was highest in the most apical 15 mm, and decreased to very low levels in the more mature root zones. By contrast, expression of *RpEXPA8*, *RpEXPA10*, and *RpEXPA18* (aerated only) increased with distance behind the root tip. Expression of *RpEXPA18* in the more basal zones was greatly reduced by root-zone O_2 deficiency. Unlike the four other genes, *RpEXPA15* did not show a clear pattern in expression as a function of distance behind the root tip.

In order to compare the expression levels of the five α-expansin genes within the various organ and tissue types, an additional real-time RT-PCR experiment was conducted (Figure 8). In petioles of plants with roots in aerated solution, *RpEXPA10*, *RpEXPA18* and *RpEXPA8* transcript levels were higher than those of *RpEXPA15* and *RpEXPA1*; while *RpEXPA8* was dominant in petioles of plants in O_2-deficient solution. In the tap root, *RpEXPA18* was dominant; and although

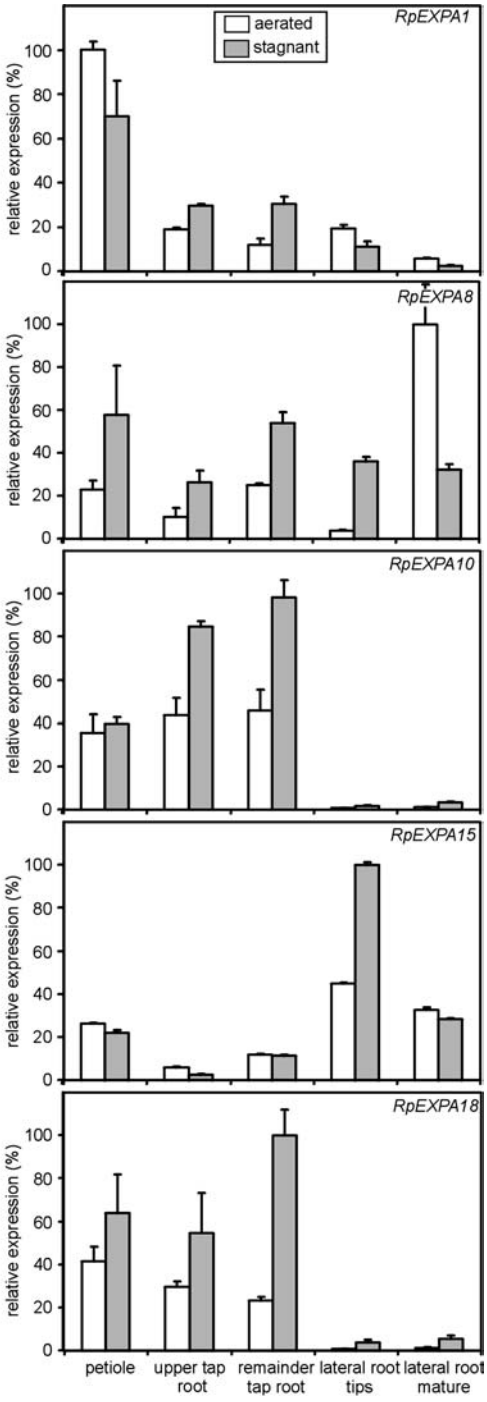

Figure 6. Relative abundances of five selected α-expansin gene transcripts in various tissues of *Rumex palustris* grown either in continuously aerated or in stagnant de-oxygenated nutrient solution for the final 10 d. Treatments were imposed on 5 week-old plants, and at harvest tissues were pooled from five replicate plants from each treatment. White bars are aerated conditions and grey bars are stagnant conditions.

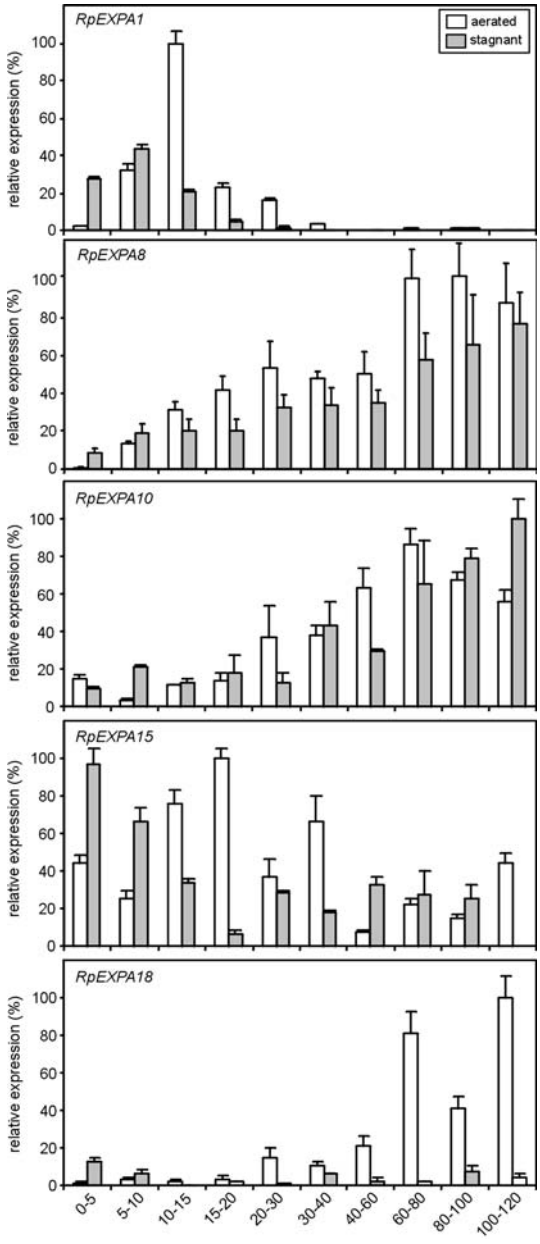

Figure 7. Spatial distributions of relative abundances of five selected α-expansin gene transcripts along adventitious roots of *Rumex palustris* grown either in continuously aerated or in stagnant de-oxygenated nutrient solution for the final 10 d. Treatments were imposed on 5 week-old plants. At harvest, tissues were pooled from five replicate plants from each treatment. Data given are means ± standard errors ($n = 2$; analytical replicates). White bars are aerated conditions and grey bars are stagnant conditions.

root-zone O_2 deficiency enhanced the relative expression of *RpEXPA10* in the upper tap root, the abundance of *RpEXPA10* was still ~4 times less

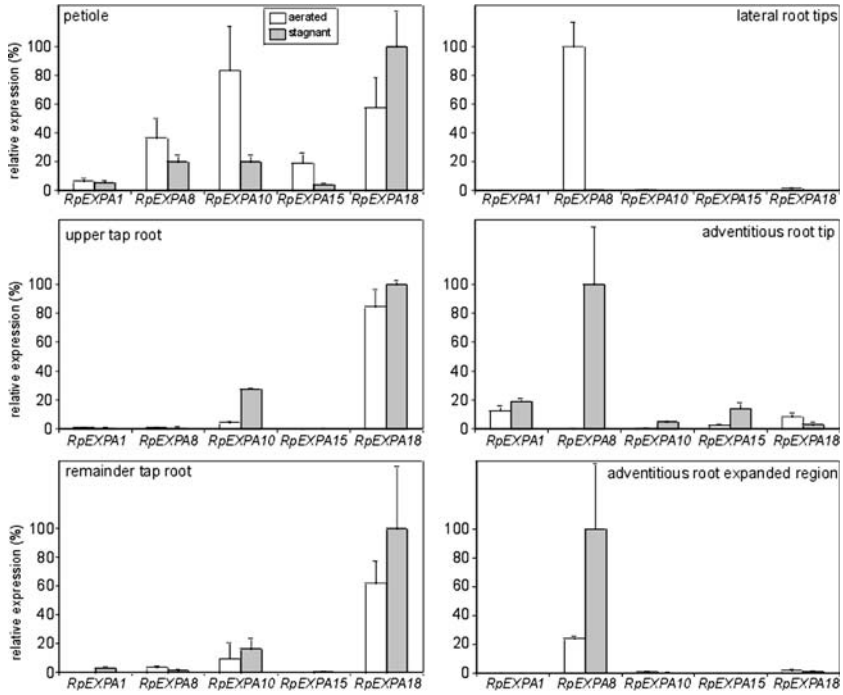

Figure 8. Relative abundance of *RpEXPA1*, *RpEXPA8*, *RpEXPA10*, *RpEXPA15* and *RpEXPA18* in several tissues of *Rumex palustris*. Plant were grown either in continuously aerated or in stagnant de-oxygenated nutrient solution for the final 10 d. Treatments were imposed on 5 week-old plants. At harvest, tissues were pooled from five replicate plants from each treatment. Data given are means ± standard errors ($n = 2$; analytical replicates).

than *RpEXPA18*. In contrast to the relatively low abundance of *RpEXPA8* in the tap root, transcripts of this gene were by far the most abundant in tips of lateral roots in aerated conditions. For tips of lateral roots in O_2-deficient conditions, *RpEXPA8* expression declined almost 100-fold. In tips of adventitious roots, *RpEXPA1* and *RpEXPA18* were co-dominant under aerated conditions, whereas in stagnant conditions *RpEXPA8* transcripts were 5-fold higher than those of the other expansins; in the expanded region of adventitious roots the difference in transcript abundance between *RpEXPA8* and the others, was even larger.

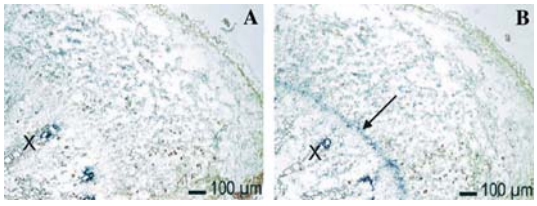

Figure 9. Localization of α-expansin mRNA in transverse sections of the upper tap root of *Rumex palustris*. (A) incubated with sense probe; (B) with anti-sense probe (see Materials and Methods). The plant was grown in aerated solution for 5 weeks and then exposed to stagnant solution for the final 72 h (magnification 48×). Specific hybridization of the α-expansin probe is evident in cells near the vascular cambium (indicated by the arrow in B). An example of a young xylem vessel, showing non-specific staining, is labeled with '×' in A.

In situ *hybridization experiments show high expression of expansins in tips of adventitious roots and near the vascular cambium of tap roots*

To assess spatial patterns of α-expansin mRNA expression within transverse sections taken from the upper 3 mm of the tap root (Figure 9) and longitudinal and transverse sections from tips of adventitious roots (Figure 10), *in situ* hybridization was applied.

In the upper tap root, specific hybridization of the α-expansin probe was evident in cells near the vascular cambium (Figure 9B). Some apparently non-specific binding (sense probe) to the walls of young xylem cells was also evident (Figure 9A).

In longitudinal sections of adventitious root tips, specific hybridization of the α-expansin probe was evident in cells near the apical meristem, and

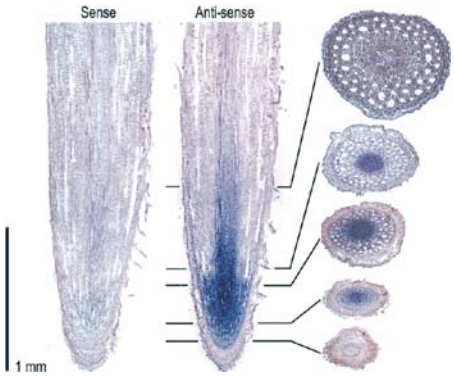

Figure 10. Localization of α-expansin mRNA in longitudinal sections of the tip of adventitious roots of *Rumex palustris* grown in de-oxygenated stagnant nutrient solution. (A) incubated with sense probe; (B) with anti-sense probe (see section 'Materials and Methods'). Specific hybridization of the α-expansin probe is evident in cells near the root tip (absent from the root cap), and within the youngest region of the stele. Several transverse sections, taken from various distances behind the tip of an adventitious root, are also shown (magnification 48×). Hybridization of the α-expansin probe was most abundant in the stele, clearly present in the cortex, but absent from the root cap. The transverse sections shown were incubated with anti-sense probe (see section 'Materials and Methods'). Transverse sections incubated with sense probe did not show hybridization (not shown).

within the youngest regions of the stele (Figure 10). However, hybridization was absent in the older regions of the stele, the cortex, the epidermis, and the root cap. Similar patterns of hybridization were observed in root tips from the aerated treatment (not shown). In transverse sections, specific hybridization of the α-expansin probe was most intense, as expected from the longitudinal sections, in the most apical mm and youngest stele tissues (Figure 10). Transverse sections were also taken from much older segments (e.g. at 10 mm behind the tip) of several roots, but α-expansin was not detected by *in situ* hybridization in any of these sections (not shown).

Discussion

Expansins are present in plants as large gene families, presumably enabling specific spatial and temporal gene regulation (Lee *et al*., 2001). *R. palustris* responds to waterlogging, by forming large adventitious root systems and enhanced development of schizogenous aerenchyma (Visser *et al*., 2000). A role for expansins has been hypothesized in both these processes (Hutchison *et al*., 1999; Jackson and Armstrong, 1999). Moreover, the high plasticity of root development shown by *R. palustris* upon waterlogging, makes this an interesting model system to study tissue-patterns of expansin gene expression.

Nineteen α-expansin genes were isolated from *R. palustris* (Vriezen *et al*., 2000; present study), all possess sequence characteristics as defined in Li *et al*. (2002). These genes show high homology on the predicted protein level, and in some cases (*RpEXPA13* and *RpEXPA14*) the only differences are in the signal peptide sequence. Twelve of the new α-expansin genes (*RpEXPA7-18*) were isolated from roots, showing that a relatively large number of expansins are expressed in the complex root system of *R. palustris*, and one (*RpEXPA19*) was isolated from petiole RNA. A similarly large number of expansin genes (*viz*. 13) were expressed in roots of *Oryza sativa*, and five of these were 'root-specific' as assessed using RNA gel-blot analysis (Lee and Kende, 2002).

In the present experiments on *R. palustris*, five α-expansin genes were selected for in-depth expression studies in various organs/tissues, and for plants exposed to aerated or O_2-deficient root-zones. Several cases of differential expression of α-expansin genes in the various root types of *R. palustris* were documented. In some root tissues, transcript levels of one gene were 4 orders of magnitude higher than others (Figure 8). Moreover, the identity of the dominant transcript differed between the root types; for example, *RpEXPA18* transcripts were high in the tap root, but were very low in tips of lateral roots; whereas, the opposite pattern occurred for abundance of *RpEXPA8* transcripts, being low in the tap root and high in lateral roots (aerated only) (Figure 8). In addition to differences between root types, environmental conditions also influenced expression of some α-expansin genes. In tips of adventitious roots, *RpEXPA1* and *RpEXPA18* were co-dominant under aerated conditions, whereas in stagnant conditions *RpEXPA8* transcripts were about 5-fold higher than *RpEXPA1* and 2 orders of magnitude higher than *RpEXPA18* (Figure 8). The present findings for *R. palustris* of a large α-expansin gene family whose members are expressed differentially in various organs and in response to low O_2, support the hypothesis by Lee *et al*. (2001) that large gene families of α-expansins

enable flexibility in expression for various organs and tissues as a normal part of development, as well as in response to abiotic stresses.

Serial sections were used to study the spatial distribution of expansin expression along adventitious roots of *R. palustris* (Figure 7). This analysis showed that *RpEXPA1* abundance was greatest in the apical 15 mm, whereas the expression of others (viz. *RpEXPA8*, *RpEXPA10*) increased substantially in the more mature zones. A similar approach using serial sections of *Zea mays* roots (Wu *et al*., 2001b), showed that two α-expansin genes were predominately expressed in the elongation zone (i.e., apical 10 mm), whereas one was expressed behind the elongation zone (i.e., 10 – 20 mm). This expression of expansins behind the elongation zone might be involved in root hair formation (Cho and Cosgrove, 2002). Relatively high expression even further back along roots, such as *RpEXPA8*, *10* and *18* in adventitious roots of *R. palustris*, might be related to formation of lateral root primordia, as root primordia show high levels of expansin mRNA, at least in rice (Cho and Kende, 1998).

In situ hybridization experiments with adventitious roots of *R. palustris* showed abundant expression of expansin mRNA in the most apical 1 mm. Similarly, expansin mRNA expression was also most abundant in the apical 1 mm of the seedling root of *Oryza sativa* (Cho and Kende, 1998) and *Glycine max* (Lee *et al*., 2003). Expression of expansin mRNA in cells soon after being produced by the root apex would be required to enable translation and localization of the protein product to enable wall loosening in the cell elongation zone. Expansin protein has been detected, using immunolocalization techniques, in the walls of cells just behind the quiescent center of the seedling root of *Zea mays* (Zhang and Hasenstein, 2000). Of the five expansin genes studied, *RpEXPA8* increased markedly in tips of adventitious roots grown in O_2-deficient solution (Figure 8), but there was no clear difference in colour development between *in situ* hybridizations with longitudinal sections of root tips grown in aerated or stagnant solutions.

For the tap root, *in situ* hybridizations of transverse sections from the upper part showed expansin expression was localized to cells near the vascular cambium (Figure 9B). This being the first report of expansin expression as associated with secondary growth in roots. The real-time RT-PCR experiment showed *RpEXPA18* transcripts were highly abundant in tap roots, followed by *RpEXPA10* (Figure 8). The *RpEXPA18* gene is therefore a candidate explaining the high expansin expression near the vascular cambium (Figure 9B), although we can not rule out that non-studied expansins could also be involved.

An important adaptation of *R. palustris* to growth in wetland environments is the formation of aerenchyma, as shown for the tap root (Figure 5 and Table 2) and for adventitious roots (Figures 3A and 4 and Table 2). Although some expansin genes are expressed at relatively high levels in tap roots (Figure 8), expression was not obvious in the outer cortex where schizogenous aerenchyma formed (Figure 9B). Since we used a non-specific probe, this raises serious doubts about any direct involvement of α-expansins in formation of schizogenous aerenchyma in the tap roots.

Aerenchyma in adventitious roots of *R. palustris* begins to form within 250 μm of the root tip (Figure 5). At this position, expansin mRNAs are already abundantly expressed (Figure 10), so expansins could be involved in this process. However, expression of expansins very close to the tip is a common feature of roots (references given above), even when aerenchyma is not formed, presumably to enable root cell elongation. Thus, aerenchyma formation in adventitious roots could not be resolved spatially from the usual cell expansion in the elongation zone. Multiple knockouts for expansin genes might provide information on their potential role(s) in aerenchyma formation. Unfortunately, the current model plant species used for molecular genetics, arabidopsis and rice, will not be useful for assessing the role of expansins in schizogenous aerenchyma development; arabidopsis does not form aerenchyma and rice forms lysigenous, rather than schizogenous, aerenchyma.

In summary, 19 α-expansin genes were isolated from *R. palustris* (Vriezen *et al*., 2000; 13 in the present study). Five genes were selected for expression analyses using real time RT-PCR. These five α-expansins showed differential expression in various root types, tissues within root types, and in response to low O_2; findings in support of the hypothesis by Lee *et al*. (2001) that α-expansin gene families enable flexibility in expression during normal plant development, as

well as in response to abiotic stresses. In the tap root, expression of expansins was localized to cells near the vascular cambium, this being the first report of expansins associated with secondary growth in roots; however, expression was not observed in tissues forming schizogenous aerenchyma. In adventitious roots, honeycomb-type schizogenous aerenchyma had already began to form within the elongation zone; i.e., aerenchyma formation and the usual cell elongation were not separated spatially, so that although expansin expression was abundant in this region, a possible role in aerenchyma formation could not be resolved. Moreover, the enhanced gas-space formation within adventitious roots of *R. palustris* in response to O_2 deficiency was due to increased divisions of some cells in radial rows in the cortex, rather than due to differential cell expansion.

Materials and Methods

Plant material and treatments

Rumex palustris plants used to isolate RNA from the upper tap root for construction of the cDNA library were germinated and grown in aerated hydroponics as described by Visser *et al.* (1996b), except that the temperature after the initial 7 d was 19 °C instead of 22 °C. Five week-old plants were transferred to stagnant, de-oxygenated nutrient solution containing agar (0.1%, w/v); the agar prevents convection in the solution. After 48 h, the uppermost 5–7 mm of the tap root was excised from each of 21 plants using a razor blade. The tissues were frozen in liquid N_2 and stored at −80 °C until RNA was extracted (described below).

In all other experiments, *R. palustris* plants were also grown in hydroponics (solution composition given in Visser *et al.*, 1996b). Seeds were sown on a ~50 mm layer of polyethylene beads floating in the nutrient solution within a plastic container (12 h at 25 °C with light at 70 μmol m^{-2} s^{-1} and 12 h at 10 °C in darkness). After 7 d, the plastic container was transferred into a controlled environment room (16 h light at 200 μmol m^{-2} s^{-1} and 8 h in darkness, constantly at 20 °C). Fourteen days after sowing, seedlings of uniform size were transplanted into individual foam holders and held within the lid of a 32 l tub containing aerated nutrient solution; there were 24 plants per tub. The pH of the solution in each tub was adjusted to 5.8 every 2 d, and each solution renewed every 7 d. To avoid shading as the plants grew, the number of plants in each tub was thinned to 12 at the end of the 4th week. Root-zone O_2 treatments were imposed on 5-week-old plants (at this time plants had seven visible leaves). The lids holding the plants were transferred to new tubs containing fresh nutrient solution, either continuously aerated or de-oxygenated (flushed overnight with N_2 gas) and then left stagnant. The plants were positioned in the foam holders so that the root/shoot junction and the bases of the petioles of the older leaves were in the solution. Various tissues were sampled at selected times for a variety of measurements Several batches of plants were grown in series (2 weeks apart) under identical conditions to provide enough tissues and replicates for the various measurements. Plant parts used for RNA extractions were excised, the same tissues from several plants in each treatment were pooled (numbers of plants used are given in the caption of each figure and table), samples were frozen in liquid N_2 and stored at −80 °C until use. Parameters of growth, development, and porosity in different root types and tissues were measured on fresh tissues, with the numbers of replicates given in the caption of each figure and table. Plant parts used for *in situ* hybridizations were fixed and embedded as described below.

Measurements of plant growth, development, and porosity

Development of adventitious roots, their extension rates, changes in diameter with distance behind the apex, and spatial distribution of porosity were evaluated for *R. palustris* grown in aerated nutrient solutions or de-oxygenated stagnant nutrient solution containing 0.1% (w/v) agar. Porosity in the tap root and primary laterals was also measured. Root diameters were measured using a microscope with a calibrated eyepiece reticule. Porosity (% gas volume per unit root volume) was measured using the method of Raskin (1983) with equations as modified by Thomson *et al.* (1990).

Anatomy of tap roots and adventitious roots

Tissues were harvested and fixed for at least 24 h in Karnovsky's fixative (Karnovsky, 1965). After

rinsing three times with water the tissues were dehydrated in ethanol solutions (50%, 70% and two times 96%) and infiltrated with Leica historesin (1:1 in ethanol, 2×1 h, and 18 h, Leica historesin embedding kit 7022 18500, Germany). Tissues were positioned in gelatin capsules and left to harden overnight. Sections of 2 μm were made using a glass knife on a Reichert OMU3 ultramicrotome and placed on pre-cleaned microscope slides (Menzel-Gläser, Germany). The sections were stretched in water and dried at 80 °C for at least 1 h, stained with 0.5% toluidine blue in water for 10–20 s and mounted in Eukitt mounting medium (EMS, USA). Micrographs were taken on an Olympus BX50 WI microscope equipped with a digital camera (Sony DKC5000)

RNA and DNA manipulations

Total RNA to be used for cDNA library construction was isolated using the acid guanidium thiocyanate–phenol–chloroform extraction method according to Chomczynski and Sacchi (1987). Poly A^+ mRNA was isolated from the total RNA sample using the Oligotex poly A^+ mRNA isolation kit (Qiagen, Hilden, Germany). Annealing was performed during 1 h at 18 °C instead of 10 min at room temperature. The library was constructed using 3.8 μg poly A^+ mRNA in the λZAPII cDNA synthesis and cloning kit and phage packaging system (gigapack III) according to the manufacturer's instructions (Stratagene Europe, Amsterdam). The unamplified library was plated, plaquelifts were made on Hybond N filters and probed with a radioactive labeled *RpEXPA1* cDNA [random primed (Feinburg and Vogelstein, 1984), α-[^{32}P]dCTP (MP Biomedicals, Amsterdam, The Netherlands), specific activity 110 TBq/mmol]. Following overnight hybridization blots were exposed to BioMax MR (Kodak) overnight with intensifying screens at −80 °C. All the positive clones were picked, replated, retested with a similar probe and if still positive *in vivo* excised according to the Stratagene manual. Plasmid DNA from the positive clones for restriction mapping was isolated using the alkaline lysis method described by Sambrook *et al.* (1989) and plasmid DNA for sequencing was isolated using the Plasmid Mini Kit (Qiagen, Hilden, Germany). Sequencing was by either the Wageningen University or the Utrecht University sequencing facility, both facilities use the dideoxy-mediated chain-termination method (Sanger *et al.*, 1977).

RNA for real time RT-PCR analysis from the various plant materials was isolated according to Kiefer *et al.* (2000) using Nucleon Phytopure DNA extraction resin (Amersham Biosciences). Residual genomic DNA was broken down with several treatments with RNase-free DNaseI (Amersham Biosciences). First strand cDNA synthesis used: $\sim$1 μg of total RNA, random hexamernucleotides (Roche Diagnostics Nederland, Almere, The Netherlands) and M-MLV reverse transcriptase (superscript III), according to the manufacturer's instructions (Invitrogen). The reaction was performed in an Eppendorf master cycler gradient programmed to the following conditions; 5 min at 25 °C and then 60 min at 50 °C, with the reaction terminated by heating to 70 °C for 15 min. Thereafter the cDNAs were diluted to 100 μl with water and 5 μl was used per RT-PCR sample.

In silico *DNA manipulations for predicted amino acid sequence analyses*

Raw sequence data were analyzed using several modules of DNASTAR software. Prediction of amino acid sequences used the same software. Signal peptide prediction was carried out using the SignalP program at http://www.cbs.dtu.dk/services/SignalP-2.0/ (Nielsen *et al.*, 1997). Alignment and phylogenetic analysis of amino acid sequences predicted from *R. palustris* expansin c/gDNAs were performed with the MEGALIGN module (DNASTAR software) using the Clustal method with the PAM250 residue weight Table and default parameters.

Analysis of gene expression by real time quantitative RT-PCR (QPCR)

A 5 μl aliquot of cDNA (50 ng) was quantitatively analyzed for the expression of each gene by the fluorogenic 5′- nuclease PCR assay (Livak *et al.*, 1995). Specific primers and probes for each gene were designed by Primer Express software (Applied Biosystems). Primer and probe sequences and modifications are shown in Table 1

and were obtained from Isogen (Maarssen, The Netherlands). Cross amplification within the primer/probe and cDNA pool was tested and was shown to be absent (data not shown). Gene-specific PCR products were continuously measured by means of an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) or Biorad MyiQ single color real-time PCR detection system. The chemicals used were as recommended by Applied Biosystems and Biorad, respectively, and the PCR reaction conditions were 2 min at 50 °C, 10 min at 95 °C and thereafter 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Each PCR assay was performed twice. A cDNA for 18S ribosomal RNA isolated from a *R. palustris* cDNA library was used as an internal sample control. For each gene the optimal primer/probe concentration was determined according to the manufacturer's instructions (Applied Biosystems Taqman universal PCR master mix protocol). For *RpEXPA1* and *RpEXPA10*, 200 nM proved to be the optimal probe concentration; for *RpEXPA8*, *RpEXPA15* and *RpEXPA18*, this was 100 nM. The optimal primer concentration was 900 nM in all cases. For the Rp18S gene, the optimal concentrations of primers and probe were 80 and 175 nM, respectively.

Post-QPCR calculations to analyze relative gene expression were performed according to the $2^{-\Delta\Delta C}{}_{T}$ method as described by Livak and Schmittgen (2001). The data for the expansin genes shown in the figures and tables are after normalization with expression data of the same sample with the 18S rDNA primer/probe combination.

In situ *hybridization*

Tissues for *in situ* hybridization were excised and immediately fixed in a solution consisting of 50% ethanol, 5% acetic acid and 3.7% formaldehyde in PBS (Drews, 1998). The procedure of embedding in Paraplast was essentially performed according to Drews (1998). Xylene-substitute (Electron Microscopy Sciences, Washington, PA, USA) was used instead of xylene. Ribbons of 7 μm thick sections were cut using a Leitz Minot microtome type 1212 and left to float on water on superfrost microscope slides (Esco scientific company, USA). The sections were 'baked' onto the slides overnight in an oven at 42 °C.

Sections were deparaffinized and rehydrated according to Drews (1998) and subsequently hybridized with digoxygenin-11-dUTP labeled sense or anti-sense probes (described below).

To visualize all the expansins present in the tissue we used a general probe which hybridizes to all other known *Rp* expansins. The template used for the probe to visualize expansins was the full length of *RpEXPA15* cDNA. The vector (pGEM-T easy, Promega) plus insert was linearized with either *PstI* (sense, T7) or *NcoI* (antisense, SP6). All restriction enzymes used were from Fermentas (Germany). Run-off transcripts were prepared using the DIG RNA labeling kit (SP6/T7) and Digoxygenin-11-dUTP (Roche Diagnostics Nederland, Almere, The Netherlands) as a label. The sense strand was generated by T7 RNA polymerase and the antisense strand was generated by SP6 RNA polymerase. The probes were tested using DIG quantification test strips according to the manufacturers instructions (Roche Diagnostics Nederland, Almere, The Netherlands).

Hybridization, and post-hybridization procedures, were essentially performed according to Drews (1998). Slides with identical sections were incubated with the anti-sense probe for transcript detection and the sense probe as a control. Visualization of the labeled probe in the tissue was performed by using anti-digoxigenein-AP Fab fragments (Roche Diagnostics Nederland, Almere, The Netherlands) and NBT/BCIP ready to use solution (Sigma). The colour was allowed to develop for several days, indicating the relatively low abundance of the expansin transcripts. Micrographs were taken on an Olympus BX50 WI microscope equipped with a digital camera (Sony DKC5000).

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