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LRX1, a chimeric LRR/extensin protein required for root hair morphogenesis in *Arabidopsis thaliana,* defines a new family of cell wall proteins

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1. Summary

In plants, the cell wall is a major determinant of cell morphogenesis. Cell enlargement depends on the tightly regulated expansion of the wall, which surrounds each cell. The mechanisms controlling qualitatively and quantitatively cell wall enlargement are still poorly understood. A new family of genes, initially discovered by computer analysis, encodes chimeric proteins containing a leucine-rich repeat and an extensin domain. Leucine-rich repeats (LRRs) are known to mediate protein-protein interactions in many eukaryotic cellular processes whereas extensins are typical cell wall structural proteins. LRR/extensins (LRXs) are encoded by a multigenic family of eleven members in *Arabidopsis* and are also present in Solanaceae (tomato) and in monocots (maize). Throughout the entire family, the LRR domain is highly conserved at the protein level whereas the extensin domain shows a wider variability. Expression studies indicate three distinct groups of *LRX* genes with similar expression patterns correlated with a strong homology of the LRR domain. The sequence similarities and chromosomal location of most of the *LRX* genes suggest that the *Arabidopsis LRX* family has evolved by gene duplication during ancient events of polyploidization. The major part of this thesis essentially focuses on the molecular and functional characterization of *LRX1*, the first *Arabidopsis LRX* gene to be identified. *LRX1* is expressed in root hair cells and the protein is specifically localized in the wall of the hair proper in which it is insolubilized during development. *lrx1* null mutants, isolated by a reverse-genetic approach, develop root hairs that frequently abort, swell and branch. The cell wall ultrastructure of the mutant root hairs displays localized defects which might result in the phenotype observed. Complementation and overexpression experiments using modified LRX1 proteins indicate that the interaction with the cell wall probably requires the extensin domain and is important for LRX1 function. The phenotypic analysis of double mutants generated between *lrx1* and other root hair mutants indicates that *LRX1* mostly acts in parallel to the other genes involved in root hair morphogenesis and that *LRX1* expression is under tight control during root hair growth. The analysis of a

second *LRX* gene (*LRX2*) was also undertaken. *LRX2* is the closest relative to *LRX1* in the *Arabidopsis* genome and both genes are likely to be paralogues. *LRX2* is mostly expressed in roots, although with a slightly different pattern than *LRX1*. *lrx2* mutants apparently do not differ from wild-type plants under the conditions which were used.

Our results suggest that LRX1 is an extracellular component of a mechanism regulating root hair morphogenesis and elongation by controlling either polarized growth or cell wall formation and assembly. LRX proteins possibly represent a novel family of plant factors involved in cell wall growth.

2. Zusammenfassung

Die Zellwand ist eine entscheidende Komponente der pflanzlichen Entwicklung. Das Wachstum der Zelle ist von einer kontrollierten Vergrösserung der Zellwand, welche jede Zelle umgibt, abhängig. Die Mechanismen, welche diesen Prozess regulieren, sind jedoch schlecht verstanden. Eine neue Genfamilie, ursprünglich durch Computeranalyse des *Arabidopsis* Genoms identifiziert, kodiert für extrazelluläre, chimäre Proteine, welche aus einer leucinreichen, repetitiven und einer Extensin-Domäne bestehen. Leucinreiche, repetitive Motive (LRRs) sind in verschiedenen eukaryotischen Systemen für Protein-Protein Interaktionen verantwortlich, wohingegen Extensine typische Zellwandproteine mit strukturellen Funktionen sind. LRR/Extensine (*LRXs*) in *Arabidopsis* sind durch eine Familie von elf Genen kodiert, wurden aber auch in Solanaceen (Tomate) und Monoktoyledonen (Mais) identifiziert. Innerhalb der *Arabidopsis* Familie der *LRXs* sind die LRR-Domänen auf der Aminosäuren-Ebene stark konserviert, die Extensindomänen jedoch variabel. Sequenzanalyse und Expressionsstudien zeigen, dass es drei verschiedene Untergruppen von *LRX* Genen gibt, deren Mitglieder jeweils ein ähnliches Expressionsmuster zeigen. Auf Grund der Homologien und der Analyse der genetischen Lokalisierung kann angenommen werden, dass die Familie der *LRX* Gene durch Genduplikation und frühe Polyploidisierung entstanden ist. Der überwiegende Teil der vorliegenden Arbeit ist auf die Charakterisierung von *LRX1* konzentriert. *LRX1* ist spezifisch in Wurzelhaaren exprimiert. Das Protein befindet sich in der Zellwand des Wurzelhaares, wo es während der Wurzelhaarentwicklung insolubilisiert wird. *lrx1* Nullmutanten, welche durch reverse Genetik isoliert wurden, zeigen Wurzelhaare, deren Entwicklung oftmals frühzeitig unterbrochen wird, die anschwellen oder sich verzweigen. Die supramolekulare Struktur der Zellwand zeigt Veränderungen, welche für den Phänotyp verantwortlich sein könnten. Komplementations- und Überexpressionsstudien mit modifizierten LRX1 Proteinen zeigen, dass die Interaktion von LRX1 mit der Zellwand von grosser Wichtigkeit ist und sehr wahrscheinlich über die Extensindomäne stattfindet. Die phänotypische Analyse

von Doppelmutanten von *LRX1* und anderen Wurzelhaarmutanten weist darauf hin, dass *LRX1* parallel zu anderen Entwicklungsprozessen des Wurzelhaares funktioniert und die Expression von *LRX1* während der Wurzelhaarentwicklung streng reguliert ist. Die Charakterisierung von *LRX2* wurde ebenfalls durchgeführt. *LRX2* zeigt starke Homologien zu *LRX1* und ist wahrscheinlich dessen Paralog. *LRX2* ist ebenfalls in Wurzelhaaren exprimiert, jedoch weicht das Expressionsmuster leicht von demjenigen von *LRX1* ab. Im Gegensatz zu den *lrx1* Mutanten scheinen sich ebenfalls isolierte *lrx2* Mutanten unter den gewählten Bedingungen jedoch nicht von Wildtyp-Pflanzen zu unterscheiden.

Zusammenfassend zeigt diese Arbeit, dass LRX1 eine extrazelluläre Komponente eines Mechanismus ist, welcher die Morphogenese von Wurzelhaaren reguliert, indem es entweder deren Spitzenwachstum selbst oder den Aufbau und die Zusammensetzung der Zellwand kontrolliert. LRX1 ist daher Teil einer neuen Familie von Proteinen, welche möglicherweise generell eine wichtige Rolle in der Entwicklung der pflanzlichen Zellwand spielen.

3. Introduction

3.1 The plant cell wall

Plant cells are surrounded by a rigid wall which mechanically supports and protects the cell from various biotic and abiotic aggressions, but also restricts cell enlargement and precludes cell migration at any stage of development. The shape and function of plant organs, therefore, largely depend on the form adopted by the individual cells they are made of. Consequently, many aspects of plant life rely on the proper regulation of cell enlargement and cell wall expansion.

The plant cell wall is a plastic and metabolically active compartment whose diverse specialized functions are reflected by its biochemical and structural complexity. Cell wall composition varies among plant species, cell types, and even within a single cell, generating domains with specific properties (Knox 1995; Freshour et al. 1996; Willats and Knox 1996; Steele et al. 1997). Current models depict the primary cell wall as an assembly of three interacting networks: cellulose-crosslinking glycans, pectins and proteins (Carpita and Gibeaut 1993). The cellulose microfibril network, conserved among plant species, forms the structural basis of the wall. A cellulose microfibril is made of several dozens of unbranched chains, condensed into paracrystalline fibers 5-15 nm wide and sometimes hundreds of micrometer long. Microfibrils are randomly organized in dividing cells or during isodiametric cell growth but orient perpendicular to the longitudinal axis during directional growth to allow turgor-driven cell expansion (Gertel and Green 1977). Cellulose microfibrils are coated and interconnected by an equal amount of cross-linking glycans which in dicots and noncommelinoid monocots (type I wall) are essentially constituted of xyloglucans. Xyloglucans are linear chains of β(1-›4) glucan with numerous xylosyl units attached on the O-6 position of the glucosyl residues. Some of the xylosyl units are linked to the glucan backbone by intermediate α-L-Ara, β-D-Gal or α-L-Fuc(1-›2)-β-D-Gal units. Xyloglucans attach tightly to the cellulose

microfibrils through hydrogen bonds and lock them together by spanning the space which separate them.

The cellulose-xyloglucan framework is embedded in a complex hydrated matrix composed of pectic polysaccharides. In dicotyledonous plants, the two main components of pectins are homogalacturonan (HGA), a helical homopolymer of (1-›4)α-D-GalA and rhamnogalacturonan I (RGI), a rod-like heteropolymer of repeating (1-›2)α-L-Rha-(1-›4)α-D-GalA units. HGA can be modified by the addition of α-D-Xyl units to form xylogalacturonans and RGI contains (1-›5)α-L arabinans, (1-›4)β-D-galactans or type I arabinans as side groups attached to the Rha units. HGAs are secreted in the apoplasm as highly methyl-esterified polymers which can then be partially deesterified by cell-wall located pectin-methyl-esterases. Deesterification allows anti-parallel HGA chains to interact at "junction zones" by cross-linking with Ca^{2+}. In addition to Ca^{2+} binding junctions, HGA molecules can form ester linkages with other HGAs or with non-cellulosic polysaccharides. The extent of esterification, the multiple cross-linking possibilities with other polymers and the modulation of the size and composition of the side chains render the pectic matrix a highly dynamic structure which can regulate the cell wall porosity and the physico-chemical microenvironment of cell wall domains and thus control the movement of enzymes and large signaling molecules in the apoplasm (Carpita and Gibeaut 1993). Pectins have a further role as a source of signal molecules involved in plant-pathogen interactions and development (Marfa et al. 1991; Ryan and Farmer 1991).

Cellulose is directly synthesized at the surface of the plasma membrane by cellulose synthase complexes which are visualized on freeze-fractured plasma membranes as hexameric clusters which were named rosette terminal complexes (Kimura et al. 1999). In the apoplasm, cellulose assembles with xyloglucans and pectins which are produced in the Golgi apparatus and secreted by vesicles.

3.2 The plant cell wall contains several classes of structural cell wall proteins

In addition to the many enzymes required for cell wall modification, cell walls contain a significant amount of structural proteins which are organized in three main classes, named according to the dominant amino acids they contain: hydroxyproline-rich-glycoproteins (HRGPs), proline-rich-proteins (PRPs) and glycine-rich-proteins (GRPs) (Keller 1993; Showalter 1993; Cassab 1998). All structural proteins are highly repetitive and apparently devoid of enzymatic activity. Most of their coding genes are differentially regulated, show tissue or cell specificity, are frequently induced upon wounding, pathogen infections, nodulation, and their transcripts are present at high levels in tissues under tensile stresses. In view of their pattern of expression, it is generally assumed that the incorporation of structural protein leads to improved mechanical properties of the walls.

PRPs and HRGPs are probably evolutionarily related and the distinction between the two becomes more difficult with the identification of new genes encoding proteins showing characteristics of both classes. GRPs also might have originated from the same ancestor as PRPs and HRGPs. Indeed, the complementary nucleotide sequences encoding proline residues can be translated into glycine residues. Therefore, GRPs might have initially evolved by inverse transcription of PRP or HRGP genes.

Glycine-rich protein cDNAs have been characterized from a variety of plants, including monocots and dicots (for review see Cassab 1998). They encode proteins containing between 50 and 70% of glycine which are arranged in Gly-X repeats (where X is frequently Gly but can be other amino acids, especially Ala or Ser). Cell wall localization has been confirmed by immunolocalization for petunia, bean and rice GRPs (Keller et al. 1988; Keller and Lamb 1989; Lei and Wu 1991; Condit 1993). GRPs are frequently expressed in vascular bundles and more specifically in xylem elements, thus colocalizing with PRPs. The presence of GRPs in cells undergoing lignification has led to the suggestion that GRPs serve as nucleation site for lignin deposition (Ye et al. 1991; Ye and Varner 1991). However, this is not supported by the data obtained on GRP1.8, which has been shown to be translated in

xylem parenchyma cells and exported to the modified primary cell wall of neighbouring dead protoxylem elements, where it is insolubilized. No correlation was found between GRP1.8 deposition and cell wall lignification (Ryser and Keller 1992). It was postulated that GRP1.8 is involved in a repair mechanism aimed at maintaining the functionality of the passively elongating protoxylem elements, either by conferring mechanical stability to the thinned wall or by forming a hydrophobic layer which would prevent water loss (Ryser et al. 1997). In experiments using GRP1.8::chitinase fusions expressed in an heterologous system, it was demonstrated that GRP1.8 interacts hydrophobically with the cell wall, a type of interaction never observed so far (Ringli et al. 2001).

Proline-rich proteins, the second class of structural proteins, were initially identified as carrot wound-induced gene products (Chen and Varner 1985; Tierney et al. 1988). Since then, many related genes have been isolated forming a vast family whose members are widely distributed in the plant kingdom. PRPs mainly contain the repetitive motif Pro-Pro-Val-Tyr-Lys with some variations, a motif which is also found in extensins (Kieliszewski and Lamport 1994). Half of the proline residues are usually hydroxylated which makes the distinction between HRGPs and PRPs somewhat artificial. However, unlike HRGPs, PRPs are not or only slightly glycosylated, probably as a consequence of the absence of $SerPro_4$ motifs. Expression of PRP genes, similar to that of many other genes encoding cell wall structural proteins, is tightly regulated during development and has been shown to be also increased by wounding, endogenous and fungal elicitors, as well as various abiotic stresses. Rapid insolubilization of the protein within the cell wall has been observed upon wounding and treatment with fungal elicitors. Although the molecular mechanism for the insolubilization of PRPs is unclear, there is evidence that it involves intermolecular oxidative cross-linking catalyzed by wall peroxidase (Bradley et al. 1992). Covalent linkage between structural proteins might result from the formation of intermolecular isodityrosine and di-isodityrosine residues (Fry 1982; Brady and Fry 1997). Ionic interactions with the acidic pectins, though, cannot be excluded since the high content in lysine of PRPs makes them highly basic. The actual function of PRPs is still unknown. Nevertheless, several roles have been postulated on the basis of their expression pattern and biochemical properties. The preferential expression of

PRPs in xylem and fibers as well as the affinity of prolyl residues for phenolic substrates suggests that PRPs might regulate the polymerization of lignin molecules. PRPs, as suggested by their rapid insolubilization upon elicitor treatment, might also be involved in pathogen defense, possibly by strengthening the cell wall (Brisson et al. 1994). A different function has been proposed for the PRP nodulin ENOD2 which is present in the nodule parenchyma. In this case PRPs might contribute to blocking oxygen diffusion by modifying the cell wall (van de Wiel et al. 1990; Bonilla et al. 1997). Genes encoding multidomain PRPs were also shown to be specifically expressed in root hairs, supporting the hypothesis that PRPs contribute to determine cell-type-specific wall properties (Fowler et al. 1999; Bernhardt and Tierney 2000). In addition, a second type of modular PRPs, possessing a C-terminal domain predicted to contain membrane spanning regions, was proposed to act as a cell wall-plasma membrane linker protein (Goodwin et al. 1996). This gene was specifically induced by cold treatment, suggesting that the protein is involved in the acquisition of cold-tolerance. Two immunologically related proteins, p33 and p36, isolated because of their interaction with the plasmamembrane in water stressed bean plants, were also shown to be PRPs (Garcia-Gomez et al. 2000).

Hydroxyproline-rich glycoproteins are the most abundant and the best known of the three classes of cell wall structural proteins. Although the term "extensin" is frequently exchanged indiscriminately for HRGPs, extensins actually form a subgroup of HRGPs, together with the arabinogalactan-proteins (AGPs) and the solanaceous lectins. Extensins were the first cell wall structural proteins to be isolated and a considerable amount of data has accumulated concerning their structure and their gene expression. In contrast, their function has not yet been clearly established. In the narrow sense, extensins are defined as basic glycoproteins containing the repeated pentapeptide $SerHyp_4$, which is frequently organized in higher order motifs. In addition to the hydroxyproline and serine residues, extensins are rich in valine, lysine, tyrosine and histidine. Most proline residues are hydroxylated and further *O*-glycosylated with short arabinosyl chains, while serine residues are glycosylated with single galactosyl units (Wilson and Fry 1986). Thus, the sugar moiety can exceed 50% of the protein mass. Structurally, extensins generally assume a polyproline II helix conformation (Wilson and Fry 1986) that confers a rod-like appearance to the

protein, as it can be visualized by electron microscopy (Stafstrom and Staehelin 1986b). This conformation is probably stabilized by the polysaccharidic side-chains (van Holst and Varner 1984; Stafstrom and Staehelin 1986a). Extensins are widely distributed among dicotyledonous plants but are less present in grasses. In the latter, classical extensins seem to be replaced by threonine-hydroxyproline-rich glycoproteins (THRGPs; Kieliszewski and Lamport 1987; Kieliszewski et al. 1990) and histidine-hydroxyproline-rich-glycoproteins (HHRGPs; Kieliszewski et al. 1992). Incorporation of Thr and His could easily have evolved from Ser and Pro, respectively, by single nucleotide exchange (AGX-›ACX, CCX-›CAX) followed by duplication of the archetypal module (Kieliszewski and Lamport 1994). However, the classical Ser-Pro_4 motif of dicot extensins is also present in the maize pollen extensin-like protein (PEX1; Rubinstein et al. 1995a; Rubinstein et al. 1995b). HRGPs constitute also the main component of the cell wall in green algae in which each different wall layer contains a distinct set of HRGPs (Adair and Apt 1990; Woessner and Goodenough 1994). In addition to their structural importance in vegetative and zygotic cells, HRGPs are also involved in the sexual mating of *Chlamydomonas* spp. Although no agglutinin gene has yet been isolated, the sexual agglutinins have the same conformation as the structural HRGPs, consisting of a rod terminated by a knob, and are similar in their amino acid composition (Adair et al. 1983; Cooper et al. 1983).

In dicots, tissue print RNA and protein blots have been extensively used to study the tissue and cell localization of several extensins. These studies revealed that the expression of extensins is under developmental control and is restricted to specific cells or tissues. However, because of the repetitive structure of most extensins, there is a risk that the nucleic acid probes or antibodies used for the specific detection of a particular species of extensin cross react with other related sequences or proteins. Although extensins are often associated with phloem and cambium, a number of extensin genes show specific expression in other cell types. A tobacco extensin is specifically expressed at the tip of emerging lateral roots (Keller and Lamb 1989), possibly reinforcing the wall of the cells submitted to a high mechanical pressure as they break through the cortex. Extensins were also found in root hairs (Bucher et al. 1997), in the style (Chen et al. 1992; Goldman et al. 1992), at the site of fungal

infection (Templeton et al. 1990) or mycorrhizal colonization (Balestrini et al. 1997). Consistently, extensin expression can be induced by fungal elicitors (Garcia-Muniz et al. 1998), wounding (Merkouropoulos et al. 1999) and in tissues which experience tensile stresses (Shirsat et al. 1996).

Extensins, which are usually recalcitrant to extraction, are produced as soluble precursors which are insolubilized in the cell wall once secreted. The mechanism of insolubilization remains unclear but it has been proposed that isodityrosine cross-links covalently bind together extensin molecules. Nevertheless, only intramolecular isodityrosines have been characterized to date, suggesting that the function of tyrosine residues is more likely to stabilize folded proteins rather than to generate an extensin network (Epstein and Lamport 1984). There is evidence that extensin insolubilization is mediated by hydrogen peroxide and could be catalyzed by extensin specific peroxidases (Bradley et al. 1992; Schnabelrauch et al. 1996). Val-Tyr-Lys motifs, which are frequent in extensins, might be the actual site of intermolecular cross-linking responsible for the insolubilization. However, other interactions with the cell wall are possible and covalent linkage between extensins and pectins was reported (Qi et al. 1995). Ionic interactions between the positively charged lysine residues of the extensins and the negatively charged uronic acids of pectins might also reversibly bind the proteins in the wall and be controlled by the pH of the apoplasm.

Our understanding of extensin function relies mainly on their structural properties and regulated expression. The main model postulates that extensins weave cellulose microfibrils, cross-link each other, directly or through the intermediate of other cell wall components, and thus lock the cell wall structure. Initially, Lamport proposed that cell expansion would require breakage of the extensin network to let the cellulose microfibrils to slide apart, which is the origin of the term "extensin". At present, this model is dismissed. The pattern of extensin expression rather suggests that their primary function is to contribute to strengthen the wall. This cell wall strengthening effect is perhaps best illustrated by the rapid cross-linking of extensins upon elicitor treatment, which renders the cell more resistant to hydrolytic cell wall enzymes (Brisson et al. 1994). In addition to a protective role, extensin might more generally be involved in development by fixing the cell shape at the end of the elongation

process (Carpita and Gibeaut 1993). However the large number and variety of extensins in plants might indicate these have different functions which depend on the cellular context. An interesting hypothesis is that extensin connect the cell wall with the plasma membrane (Knox 1995). Indeed, there is evidence that extensin interacts with transmembrane proteins and thereby stabilizes the cortical microtubules (Akashi and Shibaoka 1991).

Arabinogalactan-proteins (AGPs) form the other important sub-class of HRGPs. Unlike the other structural proteins described above, AGPs are not covalently linked to the cell wall but are usually soluble or membrane bound. Structurally, AGPs are similar to the animal proteoglycans. Only 1-10% of their molecular mass is actually constituted by the polypeptide backbone, which is rich in Hyp, Ala, Ser, Pro and Thr. The polysaccharide moiety is composed of long and highly branched galactan chains, decorated with terminal Ara units (type II arabinogalactans). Only very few genes encoding the AGPs protein moieties have been cloned so far but their analysis shows that they do not contain common motifs and are very diverse, sharing similarities with PRPs, extensins and solanaceous lectins (Chen et al. 1994; Mau et al. 1995; Du et al. 1996; Schultz et al. 2000). Recently it was discovered that AGPs can be classified into two groups: the "classical AGPs" and the "atypical" AGPs. The amino-acid composition of the protein differentiates both types, but more significantly, the "classical" AGPs contain a gycosylphosphatidylinositol-(GPI-) membrane anchor (Youl et al. 1998; Oxley and Bacic 1999; Svetek et al. 1999). Several GPI-anchored proteins from animals are implicated in signal transduction, strengthening the hypothesis which postulates that AGPs are cell surface recognition molecules involved in plant development (Du et al. 1996; Kreuger and van Holst 1996; Schultz et al. 1998; Majewska-Sawka and Nothnagel 2000). Signaling might be mediated by the phospholipase-mediated cleavage of the protein from the plasma membrane which would thus release a soluble signal in the extracellular space. Alternatively, interaction between AGPs and other membrane proteins might trigger intracellular signal transduction. A function in the organization of the growing cell walls has also been proposed (Willats and Knox 1996; Ding and Zhu 1997; Roy et al. 1999).

3.3 Leucine-rich repeat proteins are good candidates for the regulation of cell wall expansion

In the recent years several key enzymes involved in cell wall synthesis and remodeling have been identified, including cellulose synthases (Arioli et al. 1998; Taylor et al. 1999; Fagard et al. 2000; Taylor et al. 2000) and related glucosyl transferase (Favery et al. 2001), xyloglucan fucosyltransferase (Perrin et al. 1999), α-D-galactosyl transferase (Edwards et al. 1999), endo-1,4-β-D-glucanase (Nicol et al. 1998) and expansins (Cosgrove 2000). The functional analysis of some of these genes already provided insight into the way cell wall expansion proceeds. However, the molecular mechanisms that locally regulate cell wall modifications and relay information on the cell wall status to the cytoplasm, are still largely unknown. In addition to several candidates which were proposed for this role, such as AGPs or WAKs (Kohorn 2000), leucine-rich repeat (LRR) proteins, which mediate protein-protein interactions in many different cellular processes (Kobe and Deisenhofer 1994) might also have a regulatory or signaling function in the expanding plant cell wall. Leucine-rich-repeats are short sequences of usually 24 amino acids containing predominantly leucine residues and other aliphatic amino acids. They are generally organized in tandem repeats and form domains containing from 5 to 30 LRRs. In eukaryotes, LRRs are present in diverse proteins involved in protein-protein interactions (Kobe and Deisenhofer 1994). Crystallography studies of the LRR-containing porcine ribonuclease inhibitor protein and the human placental RNAse inhibitor have led to significant advances in our understanding of the structural basis of LRRs-mediated protein-protein interactions (Kobe and Deisenhofer 1995; Papageorgiou et al. 1997). Ribonuclease inhibitor proteins are made up of 15 alternating LRRs containing 28 and 29 residues, respectively. Each repeat forms an α-helix and a short antiparallel β-plated sheet separated by a β-turn. The repeats are arranged such that all the β-α hairpins have their axis perpendicular to the same plane and design the symmetric shape of a horseshoe. Whereas the α-helices define the outer face of the protein, the parallel β-sheets line the inner circumference of the horseshoe and generate the protein-protein interaction surface. The β-strand/β-turn structure is defined by the central motif xxLxLxx, where the

conserved leucine residues are buried in the protein and the flanking variable amino acids are solvent exposed and interact with the ligand (Kobe and Deisenhofer 1993; 1995). In the cocrystallized ribonuclease inhibitor-RNAse A complex, 26 out of the 28 residues involved in the interaction occur in the β-sheet region (Kobe and Deisenhofer 1996). It is likely that the conformation elucidated for ribonuclease inhibitor proteins provides a general model for many LRR-proteins. However, the number of repeats, which is highly variable from one protein to the other, influences the overall shape and curvature of the protein, as indicated by molecular modeling of decorin (Weber et al. 1996) and polygalacturonase-inhibiting proteins (PGIPs; Leckie et al. 1999).

LRR-proteins are involved in many processes as diverse as RNA processing, RNAse inhibition, binding and regulation of components of the animal extracellular matrix (ECM), cell adhesion and signal transduction. The LRR-containing proteoglycans decorin and biglycan, ubiquitous components of the ECM, bind to collagen, fibronectin and other ECM proteins, as well as to several growth factors (Kresse et al. 1997). These leucine-rich proteoglycans have an important regulatory role in the ECM assembly by controlling the formation of collagen fibrils (Iozzo 1999). Other LRR proteins such as TOLL and chaoptin are membrane-anchored adhesive proteins involved in cell differentiation and tissue organization (Krantz and Zipursky 1990; Schneider et al. 1991). Several LRR proteins are transmembrane receptor protein kinases mediating tyrosine phosphorylation upon binding of peptide hormones or growth factor. In plants, LRRs form the most common structural motifs of proteins encoded by pathogen resistance genes (R genes) and are also frequent in receptor-like kinases (RLKs). The majority of R genes cloned so far encodes cytoplasmic proteins with a nucleotide binding site (NBS) domain and an LRR domain (Ellis et al. 2000). Some R gene products, however, are transmembrane proteins with their LRR domain exposed at the cell surface and a cytoplasmic kinase domain (Jones et al. 1994; Song et al. 1995), or more frequently a short cytoplasmic tail involved in the molecular interaction with downstream signaling molecules (Hammond-Kosack and Jones 1997). Other LRR-receptor-like kinases are involved in plant development: ERECTA is implicated in the coordination of cell growth pattern and organ elongation (Torii et al. 1996); HAESA coordinates cell dehiscence in the floral organ abscission zone (Jinn et

al. 2000); BRI1 is involved in brassinosteroid perception (Li and Chory 1997; He et al. 2000) and CLAVATA1 regulates shoot apical meristem activity (Clark et al. 1997). As the best studied RLK, CLAVATA1 provides a conceptual model for RLK-mediated signal transduction (Clark 2001). Indeed, there is strong genetic and biochemical evidence that CLAVATA1 associates with CLAVATA2, an orphan LRR-receptor, and CLAVATA3, a secreted small polypeptide, which then activates the cytoplasmic kinase domain and triggers a downstream signaling cascade. CLAVATA signaling restricts stem cells to the central part of the meristem (Fletcher et al. 1999; Trotochaud et al. 2000).

Polygalacturonase inhibitor proteins (PGIPs) form another class of LRR-proteins in plants. These are soluble, extracytoplasmic defense proteins inhibiting the endopolygalacturonases secreted by fungi during infection (De Lorenzo and Cervone 1997). PGIPs isolated from different species have usually specific inhibition properties for a narrow range of phytopathogenic fungi. PGIPs are also organized in a gene family and within a single species, the different members of the family have different tissue-specific expression and substrate specificities (Desiderio et al. 1997; Devoto et al. 1997). The importance of endogenous polygalacturonases in several aspects of plant development such as fruit maturation and leaf abscission also raises the possibility that PGIPs are endogenous regulators of cell wall degradation and remodeling (Hadfield and Bennett 1998).

3.4 Root hairs as a model for cell specification and cell expansion

Plant form relies on the precise regulation and integration of cell division and cell expansion, the latter determining the definitive shape of a cell. Few models are more suitable to investigate cell specification and cell expansion in higher plants than root hairs. These are made of a single cell, are easily accessible and follow a precise morphogenetic pathway providing landmarks to assess developmental alterations (Schiefelbein 2000). Root hairs are tip-growing cells which originate from specialized rhizodermal cells (trichoblasts), which, by reorienting cell expansion, form a long tubular structure almost perpendicular to the main cell axis. Their function is to increase the contact surface between the root and the rhizosphere, thereby improving water

absorption, mineral uptake, soil anchorage and interactions with microorganisms.

In *Arabidopsis*, root hair development follows a fixed developmental pattern which can be subdivided into three steps: cell specification, root hair initiation, and root hair elongation. Root hair specification occurs early in the root apical meristem by a cell-position dependent mechanism: the immature epidermal cells located outside the anticlinal walls of the underlying cortex cells (R position) acquire the root hair cell (trichoblast) fate whereas those over a single cortex cell, and therefore in contact with periclinal wall (N position), develop into a non-hair cell (atrichoblast). This early cell specification is detected long before the hair forms because cytoplasmic differences such as vacuolation, as well as cell surface characteristics, distinguish trichoblasts from atrichoblasts shortly after cell division (Dolan et al. 1994; Galway et al. 1994). Due to the conserved cellular organization of the *Arabidopsis* root, this patterning mechanism gives rise to eight trichoblast cell files, with little variation. The current genetic model postulates that trichoblast and atrichoblast specification is determined by the relative abundance of two MYB-transcription factors, WEREWOLF (WER; Lee and Schiefelbein 1999) and CAPRICE (CPC; Wada et al. 1997). WER, which is expressed at N-position, interacts with a not yet identified bHLH protein to activate the homeodomain GLABRA2 (GL2) transcription factor which represses trichoblast cell fate. At R-positions WER is not expressed and replaced by CPC which, in association with the same bHLH protein, inactivates *GL2* expression, thus allowing root hair fate specification. The expression of these transcription factors is probably under the regulation of TRANSPARENT TESTA GLABRA (TTG), a WD-40 protein which acts early in the specification process (Galway et al. 1994; Walker et al. 1999).

However, the nature of the positional signal which specifies differential gene expression in the epidermis is still unknown. It was demonstrated that the expression of *GL2* occurs very early in the meristem during embryogenesis, indicating that positional informations are already provided at this step, but are also maintained post-embryonically to ensure the correct epidermal specification (Berger et al. 1998).

When trichoblast cells are about to reach their final length and enter the differentiation zone, root hair initiation takes place. Although trichoblast cell

specification has already occurred, the final decision of making a root hair is under the control of environmental conditions and depends on auxin and ethylene signaling. Indeed, auxin resistant mutants *axr2* and *axr3* initiate less root hairs although epidermal cells at R-position display the cytoplasmic characteristics of trichoblast cells (Wilson et al. 1990). In addition, inhibitors of ethylene biosynthesis such as aminoethoxyvinyl-Gly (AVG), or ethylene perception, like Ag^+, prevent root hair initiation (Tanimoto et al. 1995). Root hair initiation is first visible when cell wall loosening results in the formation of a small bulge at the basal part of the trichoblast (i.e. the end closer to the root tip). The position of the bulge is tightly controlled and probably requires the establishment and perception of cell polarity. Because the position of the few root hairs which develop in the mutants *rhd6*, *axr2* and *etr1* (Masucci and Schiefelbein 1996) is altered, the establishment or perception of the trichoblast polarity is likely to be under hormonal control.

Regulation of the cell wall loosening leading to the bulge formation is spatially regulated by at least two genes, *ROOT HAIR DEVELOPMENT1* (*RHD1*; Schiefelbein and Somerville 1990) and *TIP GROWTH1* (*TIP1*; Schiefelbein et al. 1993), and mutations at these loci result in enlarged bulges. Nevertheless, *rhd1* mutants form root hairs which are normal except for their swollen basis, suggesting that RHD1 function is restricted to the very early step of hair initiation. In contrast, *tip1* root hairs fail to elongate, indicating that the gene product acts also later in root hair development. In wild-type plants, the bulge formed during the initiation process subsequently elongates by tip growth. The latter represents an extreme case of asymmetric growth and relies on a precise cytoplasmic polarization, restricting exocytosis of new material to the small apical area in which the actual cell expansion occurs. Several loci implicated in the proper elongation of the root hair have been identified and can be classified into two groups, based on the phenotype caused by the respective mutations. The first group (*ROOT HAIR DEVELOPMENT2*, *SHAVEN1*, *2*, and *3*, as well as *TIP1* and *TRH1*) comprises genes required for the proper establishment of tip growth since loss-of function mutants fail to form root hairs longer than 40 μm (Schiefelbein and Somerville 1990; Parker et al. 2000; Rigas et al. 2001). The other group of genes which includes *ROOT HAIR DEVELOPMENT3* and *4* (Schiefelbein and Somerville 1990), *CAN OF*

WORMS1 (Grierson et al. 1997), *SUPERCENTIPEDE1*, *CENTIPEDE1*, *2*, *3* and *BRISTLED1* (Parker et al. 2000) is implicated in tip growth and shape determination. Mutations in those genes alter the morphology of the root hairs which either swell, branch, or wave, without preventing hair elongation. However, only three genes required for root hair morphogenesis have been cloned to date. *RHD3* encodes a GTP-binding protein acting in vacuole biogenesis (Galway et al. 1997; Wang et al. 1997), *KOJAK*, a cellulose synthase-like protein necessary for cell wall biosynthesis in root hairs (Favery et al. 2001) and *TRH1*, a potassium transporter (Rigas et al. 2001). The *rhd3* mutation is pleiotropic and results in plants with a short stature with waving root hairs, whereas the *kojak* mutation results in root hairs with a weak cell wall that ruptures soon after root hair initiation. Although *TRH1* is ubiquitously expressed, only root hairs, which fail to elongate, are negatively affected in the loss-of-function mutants.

The highly localized cell expansion confined to the root hair tip implies an asymmetric distribution of subcellular structures and molecules. Therefore, signaling is initially required to integrate intrinsic and extrinsic clues and to delimit a cortical region in which exocytosis has to take place. The secretory machinery, comprising vesicle transport and docking, has then to be established and maintained during tip growth. Although the signaling mechanism at the root hair tip remains unknown, the concentration of cytoplasmic Ca^{2+} ($[Ca^{2+}]_c$) has been shown to be a key regulator in the localization of exocytosis and sustained growth at the apex of root hairs and other tip-growing cells (Brownlee and Pulsford 1988; Garrill et al. 1993; Pierson et al. 1994; Pierson et al. 1996). Electrophysiological studies have shown that the Ca^{2+} influx is higher at the tip than at the basis of growing root hairs (Schiefelbein et al. 1992) and a steep gradient of $[Ca^{2+}]_c$ was revealed by ratiometric measurement (Bibikova et al. 1997; Felle and Hepler 1997; Wymer et al. 1997). In *Arabidopsis*, $[Ca^{2+}]_c$ reaches > 1μM in the first μm of the apex, whereas it is around 100-200 nM in the rest of the root hair. Root hairs, which have stopped to elongate at maturity or which have been treated with the Ca^{2+}-channel blocker verapamil, do not display a $[Ca^{2+}]_c$ gradient any more (Wymer et al. 1997). Consistently, the *rhd2* mutant whose root hairs fail to elongate is also unable to establish a $[Ca^{2+}]_c$ gradient after root hair initiation.

Additional evidence indicates that the $[Ca^{2+}]_c$ gradient does not merely support tip growth but contributes to determine its direction. Manipulation of the $[Ca^{2+}]_c$ gradient by photoactivation of caged calcium ionophore allows reorientation of root hairs, as does a touch stimulus (Bibikova et al. 1997). However, this reorientation is transient and growth reverts to its initial direction within 15 to 20 min, which suggests that the $[Ca^{2+}]_c$ gradient is not the primary determinant of root hair polarity but rather acts locally to organize or regulate exocytosis. Such a regulatory function might involve interactions between calcium and the cytoskeleton (Battey et al. 1999). The transport of secretory vesicles at the tip of root hairs depends on cytoplasmic movements which are caused in plant cells by actin-myosin complexes. The implication of actin in the root hair elongation is demonstrated by the growth inhibitory effect of cytochalasin D, an actin antagonist drug. F-actin is bundled into cables which extend longitudinally along the hair in cytoplasmic strands. However, these bundles flare out in the apical vesicle-rich region and are replaced by a meshwork of fine actin filaments (Braun et al. 1999; Miller et al. 1999; Baluska et al. 2000). When cytochalasin D is applied, the apical exclusion zone is invaded by thick actin filaments and concomitantly tip growth stops. Therefore actin exists in root hairs in several forms and fulfills different functions: bundled actin filaments provide tracks for myosin-dependent organelle movement whereas the apical fine actin mesh is probably involved in the delivery and fusion of the Golgi vesicles to the membrane (Kost et al. 1999; Volkmann 1999).

In contrast to the instrumental function of actin in root hair growth, microtubule implication is less clear. Microtubule interfering drugs do not prevent root hair growth but instead induce root hair shape changes such as waving and branching. Interestingly, the $[Ca^{2+}]_c$ gradient is more labile in taxol treated root hairs than in controls, and ionophore photoactivation, which locally artificially releases Ca^{2+} ions, provokes the appearance of new stably growing branches, whereas in control root hairs growth rapidly reverts to the initially defined direction (Bibikova et al. 1997; Bibikova et al. 1999). These results strongly suggest that, while microtubules are not directly involved in the elongation machinery, they are nevertheless required for the control of cell polarization and growth orientation.

3.5 Aim of the study

The existence of an intriguing new type of chimeric protein containing both an LRR and an extensin domain was first suggested by the finding that the 5' non-translated sequence of the tomato gene *TOML-4*, previously described as an extensin gene (Zhou et al. 1992), showed some homology with leucine-rich repeat domains (Jones and Jones 1997). This work was initiated to confirm this observation and initiate the molecular and functional characterization of this new type of gene. Genes analogous to *TOML-4* were identified in *Arabidopsis* and the first one to be recognized was chosen for detailed investigations using the large array of molecular and genetic tools developed for *Arabidopsis* research.

4. Results

Foreword: I would like to acknowledge the contribution of several people to the results presented or summarized below. Dr. Christoph Ringli performed the initial PCR amplification and sequencing of the 5'-end of the TOML-4 *gene. Brigitte Doesseger did the expression analysis by Northern blot and promoter-GUS plants of the* LRX3 *to* LRX9 *genes during her diploma work under my supervision, and Anouck Diet did the Northern analysis of* LRX10 *and* 11*. Finally, the cell wall ultrastructure analysis was realized in collaboration with Dr. Ulrich Ryser at the University of Fribourg where sectioning and TEM observations were done.*

4.1 Identification and description of a novel gene family encoding chimeric LRR/extensins

Computer analysis revealed that the 5'-untranslated region of the tomato extensin gene *TOML-4* (Zhou et al. 1992) has homology with leucine-rich repeat sequences. A 0.5 kb fragment spanning the putative transcription start of the gene was amplified by PCR on tomato genomic DNA and the products of three independent amplification reactions were sequenced. Comparison of the sequences we obtained by PCR, with the first published record of *TOML-4* (Zhou et al. 1992) revealed the presence of two additional nucleotides in the latter (G at position 1606 and C at position 1621, see sequence in Appendix). Since the same difference was observed in the three independently amplified products we conclude that these two additional nucleotides in the original sequence had resulted from sequencing or cloning errors. Consequently, the frame shift they introduced led to a wrong determination of the protein translation start. In order to determine the actual full-length sequence of the gene, we screened a tomato genomic library using the PCR-amplified 5'- region of the *TOML-4* gene as probe. Sequencing of the isolated clones confirmed that *TOML-4* potentially encodes a much longer protein, containing an LRR domain in frame with a carboxy-terminal extensin domain (Fig. 1). The putative protein

sequence was compared with entries in the *Arabidopsis* DNA database and the analysis revealed a total of eleven genes, encoding extensin proteins containing an LRR domain analogous to TOML-4 (the last survey was performed after the completion of the *Arabidopsis* genome sequencing). These were named *LRX* (*LRR/EXTENSIN*) and numbered according to their chronological occurrence in the database. Computer searches also identified *PEX1*, a maize pollen extensin gene as encoding an LRX-like protein. In the latter case, the identity of the N-terminal domain went unnoticed and was described as a globular domain (Rubinstein et al. 1995). *TOML-4*, *PEX1* and the *LRX* genes (collectively referred to as *LRX* family in the rest of the text) encode proteins which share the same primary structure, consisting of a predicted signal peptide, a short N-terminal domain showing no significant homology to known functional motifs, an LRR domain, and a carboxy-terminal extensin-like domain (Fig. 2). The LRR domain of each LRX protein comprises ten complete LRRs plus one truncated putative repeat at the carboxy-end of the domain. In contrast to the conserved number of LRRs, the extensin domain shows a large variability both in length and in structure (Fig. 1 and 2B and sequences in appendix).

The leucine-rich repeats contain 22 to 25 residues and match the plant extracytoplasmic LRR consensus sequence LxxLxxLxxLxLxxNxLxGxIPxx, were L represents leucine residues, frequently replaced by other aliphatic amino acids (essentially valine, isoleucine or methionine) and x indicates non conserved residues. At the amino acid level, the LRR domains of the LRX proteins have, in average, 35% of identity with the LRR domain of other characterized LRR-proteins such as CLAVATA1 or BRASSINOSTEROID INSENSITIVE1 (data not shown). However, the percentage of LRR sequence identity observed within the LRX family, which ranges from 94.5 (LRX6-LRX11) to 50% (LRX8-LRX9), distinguishes the LRX proteins as a separate and novel sub-family of LRR-proteins.

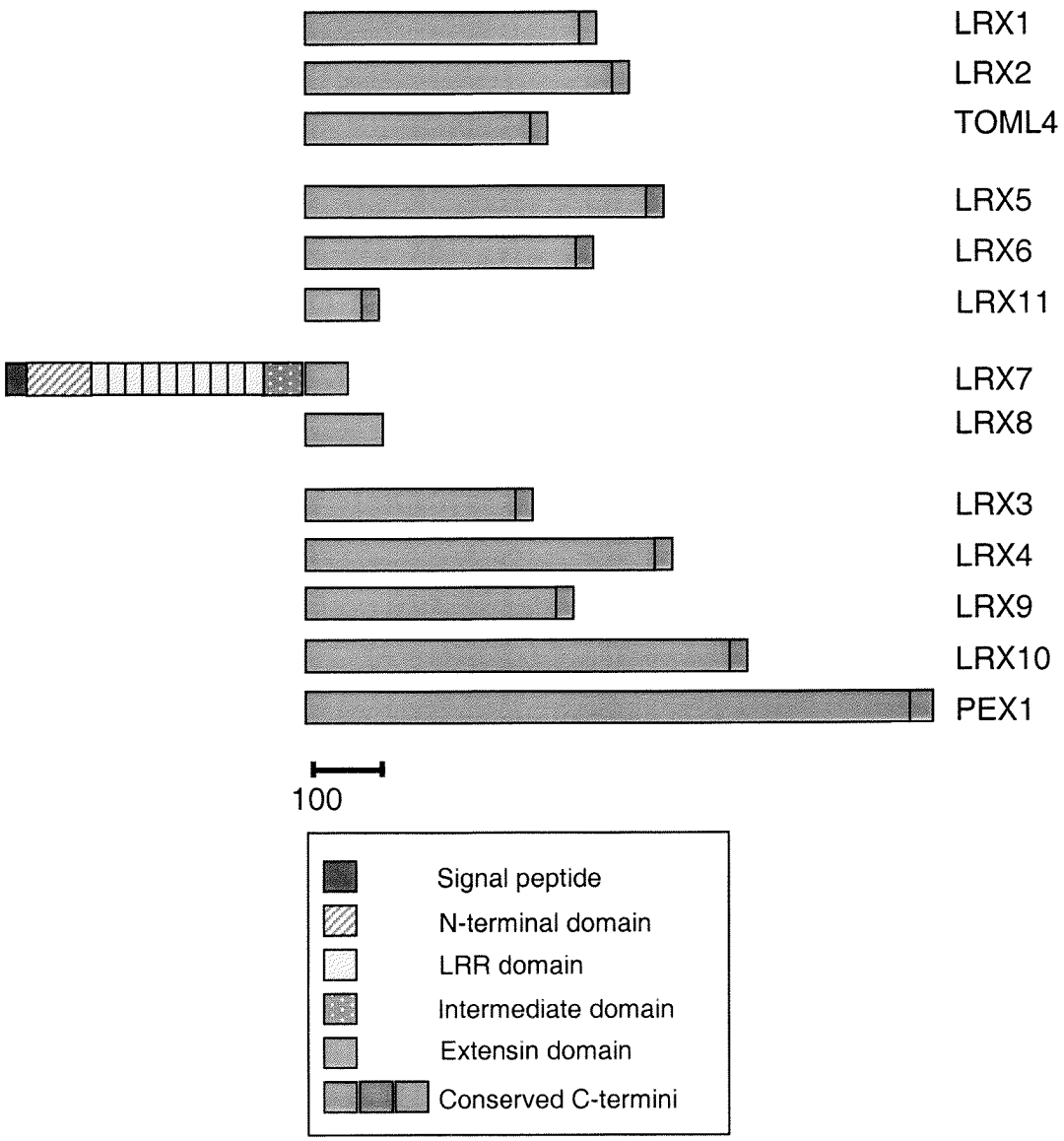
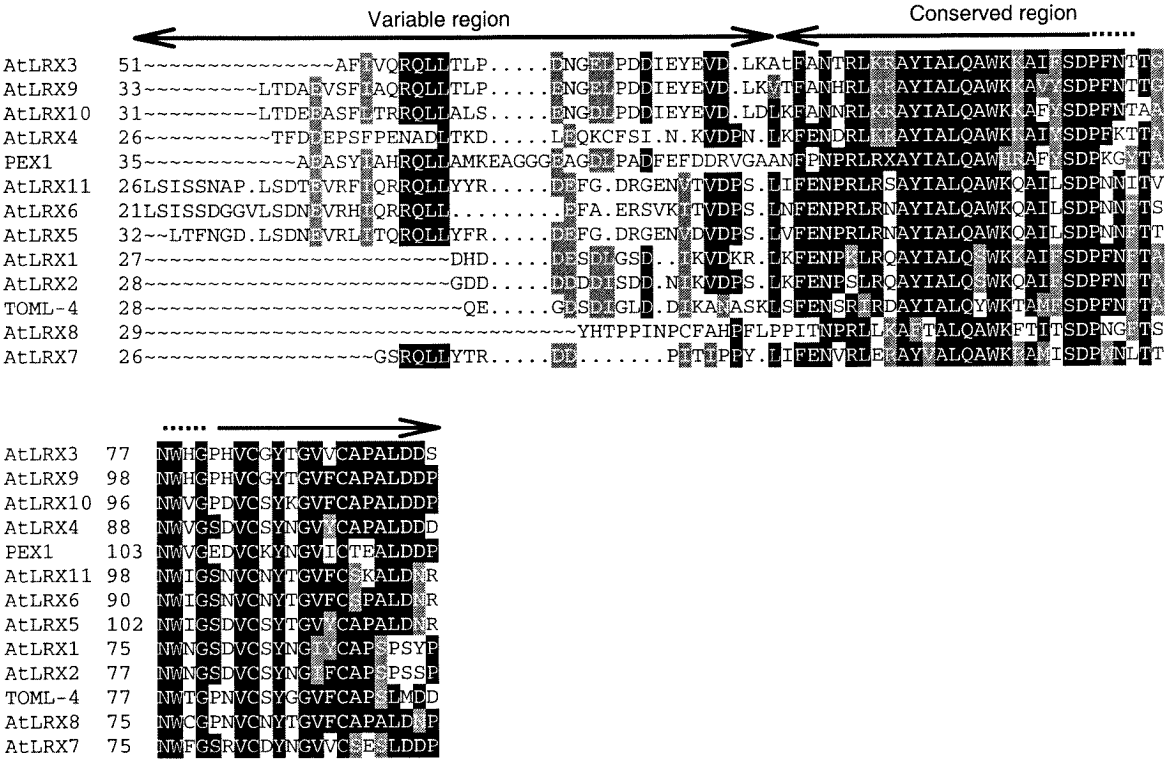
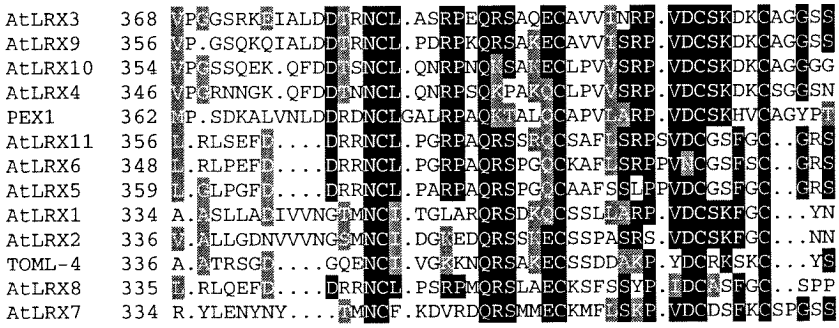


Figure 1. Schematic representation of the *LRX* gene family and similar genes in maize and tomato indicating the different domains of the encoded proteins. The signal peptide, N-terminal domain, LRR domain and intermediate domain, which are of similar size in every member of the family are represented only once. The extensin domain of each protein is displayed and groups have been formed according to the identity of the conserved C-terminus and the sequence similarity of the respective LRR domains.

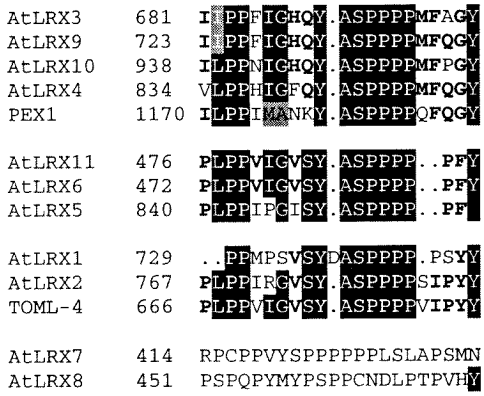
A. N-terminal domain:



C. Intermediate region:



D. C-terminal conserved motifs:



B. LRR domain:

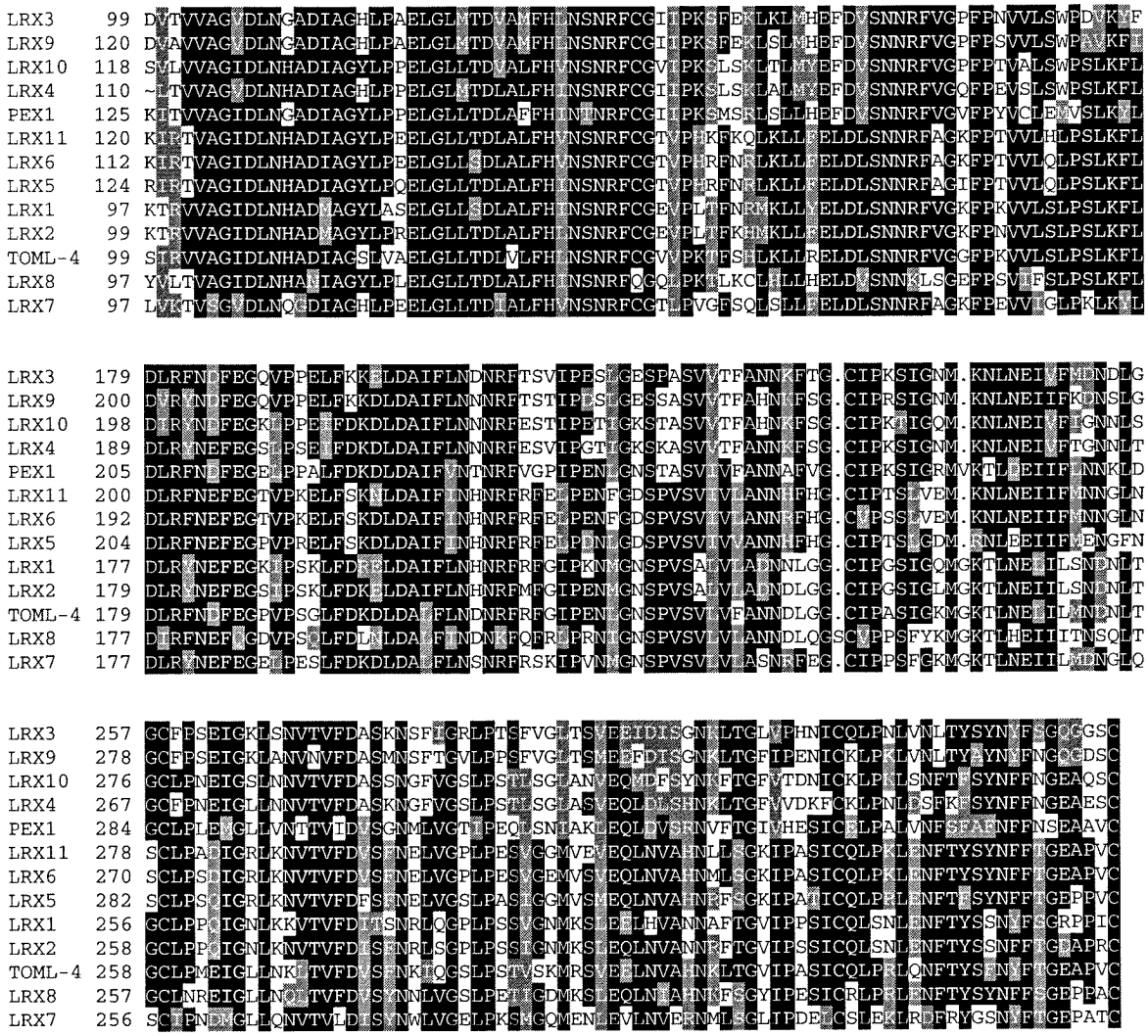


Figure 2. Amino acid alignment of the deduced protein sequences of *TOML-4*, *PEX1* and *LRX* genes. The N-terminal domain, containing a variable and a conserved region (*A*), the LRR domain (*B*), the intermediate domain (*C*) and the conserved C-termini have been aligned individually. The signal peptide was excluded from the alignment and its position is represented by the wave symbol (~). Identity and similarity in more than 50% of the analyzed genes are indicated by black and grey shading, respectively. Amino acids specifically conserved within the groups at the C-termini are represented in bold (*C*). The extensin domain has been omitted because of its strong variability.

The protein sequence relationships are best illustrated by a dendrogram representation based on the 10 complete LRRs of each LRX protein: within the *Arabidopsis* LRX proteins three subgroups can be delimited: group A, comprising LRX1 and LRX2, group B, which includes LRX3, LRX4, LRX9 and LRX10, and group C containing LRX5, LRX6 and LRX11 (Fig. 3). The sequence similarity within these groups is 88% for group A, 69% to 85% for group B, and between 85.5% and 94.5% for group C. Interestingly, the maize PEX1 and the tomato TOML-4 proteins are clustered with the *Arabidopsis* group B (62% of identity in average) and group A (74% of identity in average), respectively, instead of forming distinct branches of the tree. This likely indicates a good conservation of the LRR domains within the LRX family since the divergence between monocots and dicots (200-240 million years ago; Wolfe et al. 1989) and between asterids and rosids (90 million years ago; Gandolfo et al. 1998).

On the basis of the LRR domain alignment performed with a consensus cut off set at 0.5, 21% of the amino acids are substituted by non-similar residues. If the analysis is restricted to the regions forming the solvent exposed β-sheet in each LRR, only 10.4% of the residues are substituted, indicating a better conservation in the part of the sequence involved in the interaction with the ligand.

The N-terminal domain displays a variable region which, besides the presence of the RQLL motif in eight of the thirteen LRX proteins, has a low degree of conservation, followed by a region of high identity (Fig. 2A). The function of the conserved motifs is unknown and no homology with other proteins was found. The intermediate domain which separates the LRRs from the extensin-like domain is relatively variable but shows several conserved positions, such as three Cys residues which are perfectly conserved throughout the whole family (Fig. 2C).

The extensin domain of the LRX proteins contains the pentapeptide $SerPro_4$, which is frequently organized in longer repeats. This motif, in its hydroxylated form ($SerHyp_4$), is characteristic of extensins. However, besides an overall similarity conferred by the repetition of the $SerPro_n$ motif, the extensin domain does not show the same conservation as observed in the LRR domain. The extensin domain is highly variable in size and ranges from 59 to 700 amino acids. Its content of Pro, Tyr, Lys and its organization in higher order repeats

are also very variable. It is nevertheless possible to recognize similarities in several of the sequences, which reflect the homology found in other parts of the protein. The extensin domain of LRX1 and LRX2, for instance, can be divided into sub-domains characterized by the presence of closely related high-order repeats (compare Figs. 5 and 20). In LRX3 and LRX9, the same unique repeat (PVHKPQPPKESPQPNDPYDQSPVKFRR) is present at a similar position in the extensin domain and is followed by a long series of SP_nVH/Y repeats. Finally, at the beginning of their extensin domain, both LRX4 and LRX10 have a region rich in Gln residues and Pro-Lys doublets. The C-terminus is systematically conserved within the groups established according to LRR sequence similarity (Fig. 2D).

The distribution of the *LRX* genes in the *Arabidopsis* genome shows that *LRX1* and *LRX2*, *LRX4* and *LRX10*, *LRX11* and *LRX6*, *LRX3* and *LRX9*, respectively, consist of gene pairs located on duplicated and transposed blocks of chromosome (Fig. 4). Nevertheless, two *LRX* genes (*LRX5* and *LRX11*) have no equivalent on the other duplicated chromosome copy, whereas *LRX7* is present on a chromosome part which was not duplicated. Interestingly, the *LRX* genes found on duplicated blocks show systematically the highest sequence identity (Fig. 3).

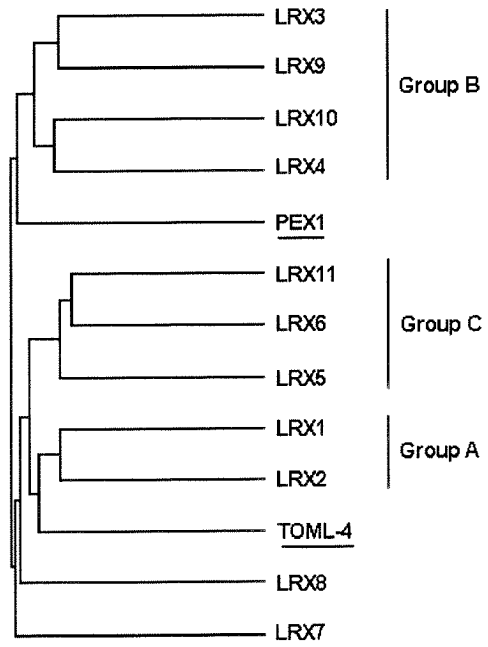


Figure 3. Dendrogram representation of the protein sequence similarity of the LRR domain (the 10 complete LRRs) in the LRX family, including TOML-4 and PEX1. Three groups of homology are indicated as groups A, B and C. Protein originating from species other than *Arabidopsis* are underlined.

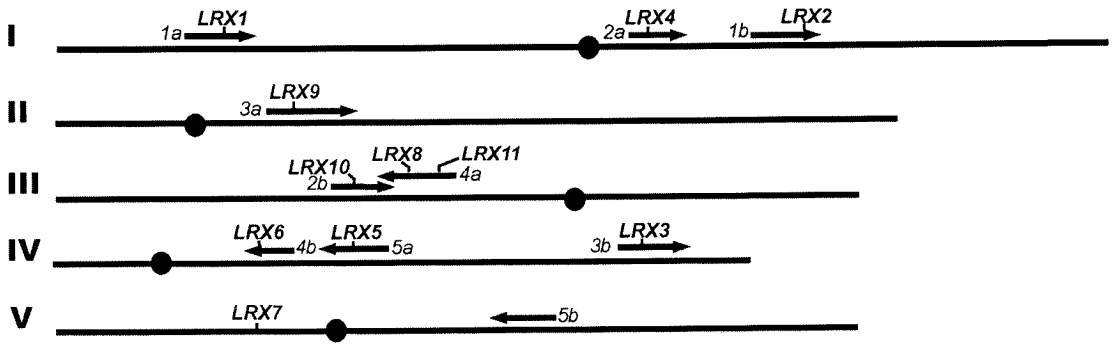


Figure 4. Chromosomal positions of the *LRX* genes and correlation with duplicated sequence blocks. Duplicated blocks are represented by arrows, each copy (a and b) of a pair having the same number. The direction of the arrowhead indicates the predominant relative orientation of the open reading frames within each block. The black lines represent each chromosome and the black dots indicate the position of the centromers. The position of each known *LRX* gene is indicated. For clarity, only the duplicated blocks containing the *LRX* genes are represented. Every *LRX* gene, except *LRX7*, is located on a duplicated sequence. However, the b-copy of block 5 does not contain any *LRX* gene although the a-copy carries *LRX5* (adapted from Vision et al. 2000).

4.2 Molecular and functional characterization of LRX1

Because *LRX1* and *LRX2* were the first two *Arabidopsis LRR/extensin* genes to be identified, the present work was mainly directed towards the molecular and functional characterization of *LRX1* and, to a lesser extent, *LRX2*.

4.2.1 Genomic and cDNA sequence analysis

LRX1 is located on chromosome I, BAC clone F12F1, and consists of an intronless open reading frame of 2235 bp, encoding a protein of 744 amino acids. The genomic sequence was physically isolated by screening a λ phage genomic library with a probe covering the LRR-coding sequence. A clone containing the open reading frame, 1600 bp of untranslated 5' sequence and 800 bp of 3' sequence was obtained and sequenced. The *LRX1* cDNA was amplified by RACE-PCR on total RNA of 2-week old seedlings. Comparison between the *LRX1* genomic sequence and the cDNA sequence confirmed the absence of introns. The positions of the transcription start and of the polyadenylation signal were localized 36 nucleotides upstream and 2442 nucleotides downstream of the ATG protein translation start, respectively (data not shown). The LRX1 protein primary structure corresponds to the general organization of the LRR/extensin mentioned above: it includes a predicted signal peptide, an LRR domain of 260 amino acids and a carboxy-terminal extensin-like domain of 363 amino acids (Fig. 5). The LRR domain of LRX1 shares 53% of sequence identity with the amino-terminal domain of PEX1 and 73% with the LRR domain of TOML-4 (Zhou et al. 1992). Numerous $SerPro_4$ motifs, which are organized in three different higher order repeat sequences (SP_5(Y/S)SKMSPSVRAY; SP_4YVYS; SP_4(S/V)P(V/L)YYPxVTx, where x is P, Q, S, N, or Y), constitute much of the carboxy-terminal domain of LRX1 (Fig. 5). The extensin domain contains nine Tyr-x-Tyr triplets, which are considered as potential sites for either intra- or intermolecular covalent cross-linking (Kieliszewski and Lamport 1994). The *LRX1* gene was found only once in the *Arabidopsis* genome sequence and Southern blot analysis confirmed that it is a single copy gene (Fig. 6).

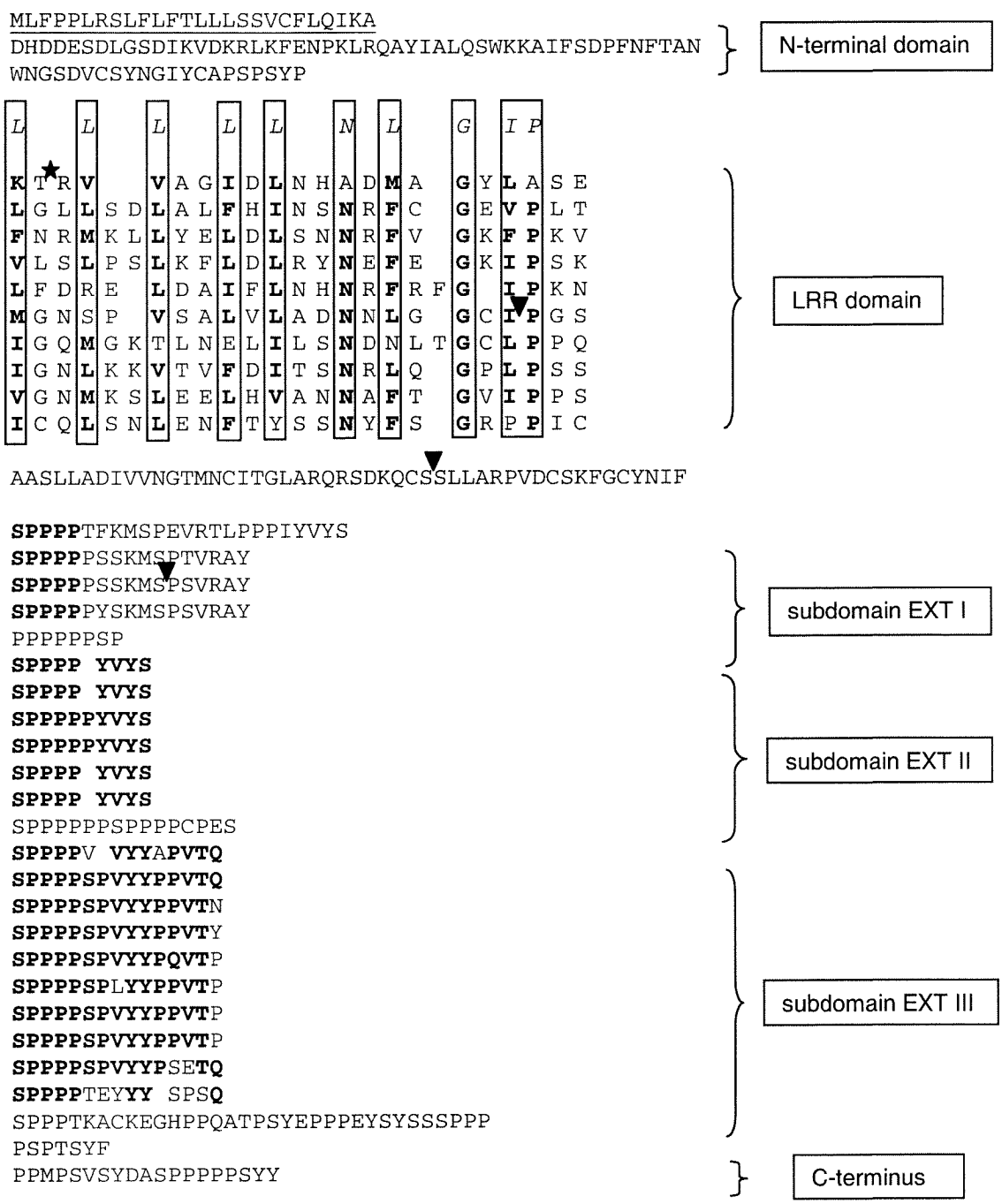


Figure 5. Deduced amino acid sequence of the *LRX1* gene. The predicted signal peptide is underlined and the different domains have been arranged to highlight their organization. The LRRs are aligned on the plant extracellular LRR consensus sequence (*first line in the frames*). The conserved amino acids are indicated in bold. The diverse higher-order repeats of the extensin domain are aligned, their limits indicated, and the amino acids conserved within those repeats are written in bold. *Black arrowheads* indicate the position of the three *En-1* insertions. The *asterisk* indicates the positions of the c-myc tag in the construct used in the immunolocalization experiments.

4.2.2 LRX1 *is expressed in differentiating root hair cells*

The expression pattern of *LRX1* was determined by Northern hybridization, RT-PCR, and analysis of transgenic plants harboring the bacterial *uidA* gene under the control of the *LRX1* promoter (*pLRX1::GUS*). Northern hybridization revealed a single band of approximately 2.6 kb exclusively present in root RNA extracts (Fig. 7A) corresponding in size to the *LRX1* cDNA obtained by RACE-PCR. The root specific expression of *LRX1* was also confirmed by RT-PCR which only allowed amplification of the *LRX1* mRNA from root extracts (Fig. 7B). The expression of the *pLRX1::GUS* construct was analyzed in 10 independent transgenic lines. Consistent with the data obtained with Northern hybridization and RT-PCR, GUS activity was only detected in roots and was first observed in the epidermal cells of the collet transition zone, where the first root hairs are formed shortly after germination (Fig. 7C, panel 1). After further development of the seedlings, expression was observed to be restricted to trichoblasts along the differentiation zone (Fig. 7C, panels 2-4). GUS activity was first detected in cells where a small bulge could be identified as an emerging root hair (Fig. 7C, panel 5) and persisted during root hair formation. In mature parts of the root, GUS activity gradually faded. No expression was detected in either the meristem region or the elongation zone of the root, whereas columella cells in the root cap were stained (Fig. 2C, panel 3). The same GUS expression pattern was reiterated in lateral roots (data not shown).

Because root hair development is dependent on and promoted by ethylene, we tested the expression of the reporter gene in *pLRX1::GUS* seedlings treated with the ethylene biosynthesis inhibitor L-α-(2-amino-ethoxyvinyl)gly (AVG; Tanimoto et al. 1995). AVG treatment almost completely prevented both root hair development and GUS expression in *pLRX1::GUS* seedlings compared to untreated control plants (Fig. 7D). Endogenous *LRX1* gene expression was also almost completely abolished in AVG-treated wild-type plants (Fig. 7E, left panel). Similarly, *LRX1* expression was strongly reduced in the almost root hairless *rhd6* mutant (Masucci and Schiefelbein 1994) (Fig. 7E, right panel). These results confirm that *LRX1* expression is highly correlated with root hair development.

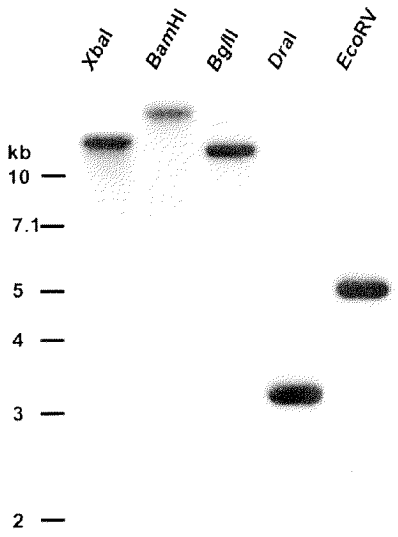
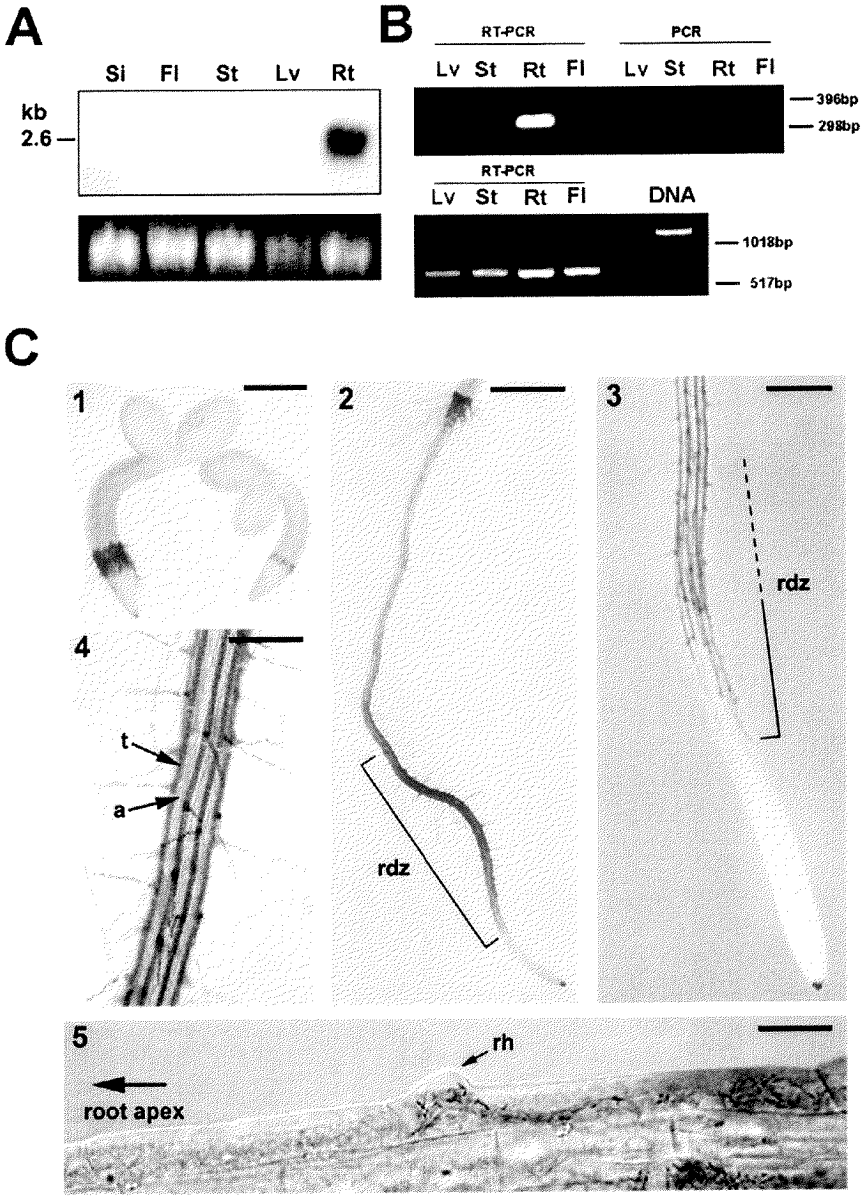


Figure 6. Southern blot of *Arabidopsis* genomic DNA hybridized with a *LRX1* specific probe. Genomic DNA digested with the restriction enzymes indicated on the top was hybridized with a ^{32}P-labelled *LRX1* probe

Figure 7. *LRX1* expression pattern. (*A*) Organ-specific expression of *LRX1*. Total RNA was extracted from green siliques (*Si*), flowers and flower buds (*Fl*), inflorescence stems (*St*), and rosette leaves (*Lv*), of 35-40-day-old wild-type Columbia plants. Roots (*Rt*) were harvested from 14-day-old seedlings grown vertically on MS medium. For Northern analysis total RNA (10 μg) was hybridized with a ^{32}P-labeled LRX1 probe and 25S rRNA was used as loading control (*lower panel*). (*B*) RT-PCR experiments with the same RNA preparations as in (*A*). *LRX1* transcripts were amplified by PCR with gene-specific primers after reverse transcription (*RT-PCR*). In order to test for DNA contamination of the RNA samples, the same experiment was made in parallel without reverse transcriptase (*PCR*). Integrity of the RNA template was checked by RT-PCR using primers specific for *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), a constitutively expressed gene (lower panel). *GAPDH* amplification from DNA template (*DNA*) yields a larger fragment because of the presence of an intron in the gene. (*C*) Transgenic seedlings containing the *LRX1* promoter fused to the *uidA* gene were histochemically stained at different developmental stages to reveal the tissue-specific expression of *LRX1*. GUS activity (blue staining) was first detected in the epidermal root hair forming cells of the collet region, 2 days after germination (*1*). In 4-day-old seedlings, GUS activity was mainly detected in rhizodermal cells of the root differentiation zone (*rdz*) and in the root cap (*2, 3*). In the differentiation zone only trichoblast cell files (*t*), which alternate with atrichoblast cell files (*a*), show GUS activity (*4*). *GUS* expression is first visible within cells undergoing root hair (*rh*) initiation (*5*). Bars, 400 μm (*1, 3*), 800 μm (*2*), 200 μm (*4*), 20 μm (*5*). (*D*) *GUS* expression in AVG-treated seedlings. Treatment with 20 μM AVG (*AVG+*) prevented root hair formation and blocked *GUS* expression compared to control seedlings (*AVG-*). Bar, 250μm. (*E*) *LRX1* expression in AVG-treated seedlings and *rhd6* mutants. Total RNA was extracted from seedlings grown on MS medium containing the ethylene biosynthesis inhibitor L-α-(2-amino-ethoxyvinyl)glycine (*left panel, AVG+*), from *rhd6* mutants, which do not develop root hairs (*right panel, rhd6*) and from wild-type control plants grown on normal MS medium (*left panel, AVG-; right panel, WT*). Total RNA (10μg) was used for Northern analysis. 25S rRNA was used as loading control (*lower panels*).


A
Si Fl St Lv Rt
kb
2.6
B
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PCR
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396bp
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1018bp
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C
1
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4
5
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a
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D
AVG-
AVG+
E
AVG- AVG+
WT
rhd6

4.2.3 Expression of the LRR domain in E. coli *and production of anti-LRX1 antibodies*

The LRX1 sequence coding for the LRR domain was amplified by PCR and cloned as a translational fusion with a 6x His tag into the expression vector pQE30. The construct was transformed into *E.coli* and the expression of the recombinant LRX1-LRR domain was monitored by SDS-PAGE of bacterial protein crude extract (Fig 8A). A prominent band at 28 kDa corresponding to the predicted size of the LRR domain confirmed the expression of the recombinant polypeptide in the bacteria. RecLRX1 was purified by metal chelate affinity chromatography on Ni-NTA agarose beads and purity of the preparation was verified by SDS-PAGE (Fig. 8B). Because dialysis of the eluate resulted in protein precipitation, the immunization of two rabbits was performed with the denaturated protein diluted in 8 M urea. The IgG fraction from serum of immunized rabbits was purified on protein A affinity column and used as such. The anti-LRX1 antibodies recognized both the purified recombinant LRX1-LRR and LRX2-LRR domains on western blots indicating that the two proteins share common epitopes (Fig. 8C). A plant polypeptide of 150 kDa (p150) was detected in crude plant protein extracts. p150 was not detectable when the antiserum was preabsorbed with the recombinant LRX1-LRR domain (recLRX1) but not when it was preabsorbed with the recombinant LRR domain of LRX2 (recLRX2) (Fig. 9A). However, p150 was also detected in mutant plants harbouring a disrupted *LRX1* gene, though with a lower intensity. These data suggest that p150 is composed of several proteins which are immunologically related to LRX1 and possibly include LRX1 itself.

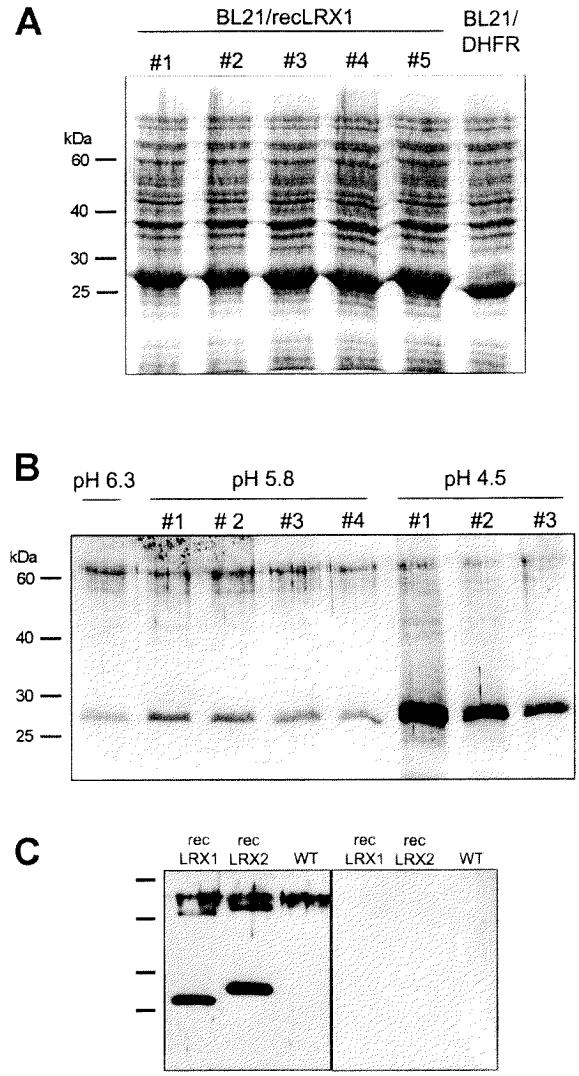

Figure 8. Expression of the LRR domain of LRX1 in *E.coli.* (*A*) Crude protein extracts of *E. coli* cultures expressing the LRR domain of LRX1 fused to a His-tag (recLRX1). As control, a strain expressing DHFR (dehydrofolate reductase) was used. (*B*) Purification of recLRX1 by metal chelate affinity chromatography and elution at decreasing pH. Release of the protein was maximal in fractions with a pH of 4.5 and these fractions were mostly free of contaminating bacterial proteins. Numbers at the top of the lanes indicate fraction order.(*C*) Reaction of the anti-recLRX1 with protein extracts from bacteria producing recLRX1 or recLRX2 (the LRR domain of the closely related LRX2) and from wild-type bacteria (*WT*). Both recLRX1 and recLRX2 were equally recognized in crude extracts. The right panel represents a replicate blot incubated with preimmune serum.

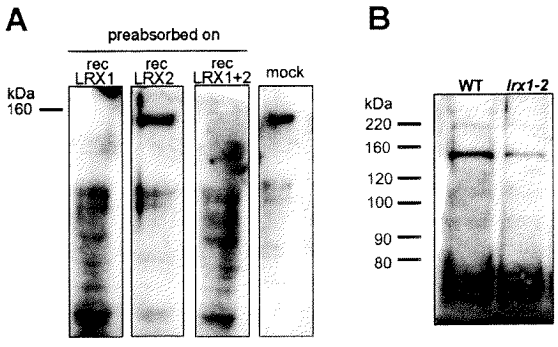

Figure 9. Western blot immunodetection of crude protein extracts from plants with the anti-LRX1 polyclonal antibodies. (*A*) Plant proteins were extracted with high-salt buffer from 2-week-old *Arabidopsis* seedlings. Before use in western blots, the antiserum was either preincubated with recLRX1, recLRX2, both recLRX1 and recLRx2, or in buffer (*mock*). The antibodies recognized a 150 kDa polypeptide in the plant extract and recognition was abolished by preabsorption of the antibodies with recLRX1. (*B*) The 150 kDa polypeptide is also present in *lrx1* null mutants (*lrx1-2*) although in smaller amount compared to wild type (*WT*).

4.2.4 LRX1 is located in the root hair proper and is insolubilized during development

To determine the cellular localization of LRX1, transgenic plants expressing a c-myc-tagged LRX1 protein under the control of the *LRX1* promoter sequence (mycLRX1 plants) were generated (see fig. 5 for the precise position of the tag). This approach was used to specifically detect LRX1, considering the number of related *LRX* genes whose products might cross-react with antibodies raised against LRX1. On protein blots, anti-c-myc monoclonal antibodies (myc-mAbs) detected a single polypeptide of 160 kDa in root protein extracts of mycLRX1 plants but not of wild-type plants (Fig. 10A). The difference between the apparent molecular mass and the calculated size of the LRX1 protein sequence (85 kDa) is probably due to extensive glycosylation of the extensin domain or to its particular conformation.

In order to investigate the organ distribution of the LRX1 protein, we performed tissue-print immunolocalization of mycLRX1 in young seedlings. In mycLRX1 plants, signals were detected along the root at positions corresponding to the differentiation zone, whereas no signal above background was detected in control plants (Fig. 10B). The tagged protein was also localized at the cellular level by whole-mount immunolabeling. Root hairs proper were specifically labeled whereas the rest of the trichoblast cells was not decorated (Fig. 10C, panels 1, 2 and 3). In agreement with the *pLRX1::GUS* expression pattern, no labeling was observed in either atrichoblast, meristematic or elongating cells (data not shown). MycLRX1 was detected throughout root hair development: during initiation (Fig. 10C, panel 3), elongation (Fig. 10C, panel 4) and even after root hair maturation (Fig. 10C, panel 5). Labeling was evenly detected all over the root hair structure, indicating a regular distribution of the protein (Fig. 10C, panel 1). Thus, whole-mount immunolocalization revealed that mycLRX1 is present specifically in root hairs at all developmental stages. However, in tissue-print analysis, mycLRX1 was detected only along the differentiation zone. This difference suggests that LRX1 is soluble in the early stages of root hair development and becomes insolubilized in later stages. As HRGPs have been shown to be insolubilized in the cell wall by oxidative cross-linking (Fry 1982; Bradley et al. 1992) the LRX1 extensin domain

might mediate the interaction of mycLRX1 with other cell wall components during root hair development.

To determine if mycLRX1 is immobilized in the cell wall, root cell walls were purified by extraction in phenol-acetic acid to remove soluble proteins as well as cytoplasmic and membrane components. The insoluble cell wall fraction was then immunolabeled with myc-mAbs. Elongated thin-walled structures, identified as root hair debris, were regularly decorated in mycLRX1 plant material (Fig. 11, left panels) whereas such structures were not labeled in wild-type material (Fig. 11, right panels). This indicates that a significant amount of mycLRX1 protein strongly interacts with the root hair cell wall and can not be extracted.

Figure 10. Identification and immunolocalization of the LRX1 protein by epitope tagging. (*A*) Protein gel blot of root extracts from wild-type (*WT*) and transgenic (*mycLRX1*) plants expressing the c-myc-tagged LRX1 protein under control of the *LRX1* promoter. Blots were incubated with the mouse myc-mAbs (*left panel*) and a duplicate gel was stained with Coomassie blue to test for equal loading (*right panel*). (*B*) Tissue print immunoblot. Four-day-old wild-type (*WT*) and transgenic mycLRX1 (*mycLRX1*) seedlings were pressed on nitrocellulose membranes to transfer soluble proteins. A scheme of a seedling at the same scale as the blots is represented on the left of the panels for orientation. *Arrowheads* indicate the position of each root tip and *frames* delimit the root differentiation zone. The dark spots at the top of the membrane were caused by anthocyanins of the cotyledons also transferred onto the membrane. Signals were visible in the differentiation zone of mycLRX1 plants (*mycLRX1*) but not in controls (*WT*). Bars, 1mm. (*C*) Whole mount immunolocalization of mycLRX1. Three-day-old wild-type (*WT*) and mycLRX1 (*mycLRX1*) seedlings were fixed and immunolabeled with myc-mAbs. The c-myc epitope was found to be associated with root hairs in mycLRX1 (*1*) but not in wild-type (*2*) seedlings. Labeling was visible from root hair initiation (*3*), throughout root hair elongation (*4*) and after root hair maturation (*5*). Bars, 100 μm (*1, 2*), 25 μm (*3-5*).

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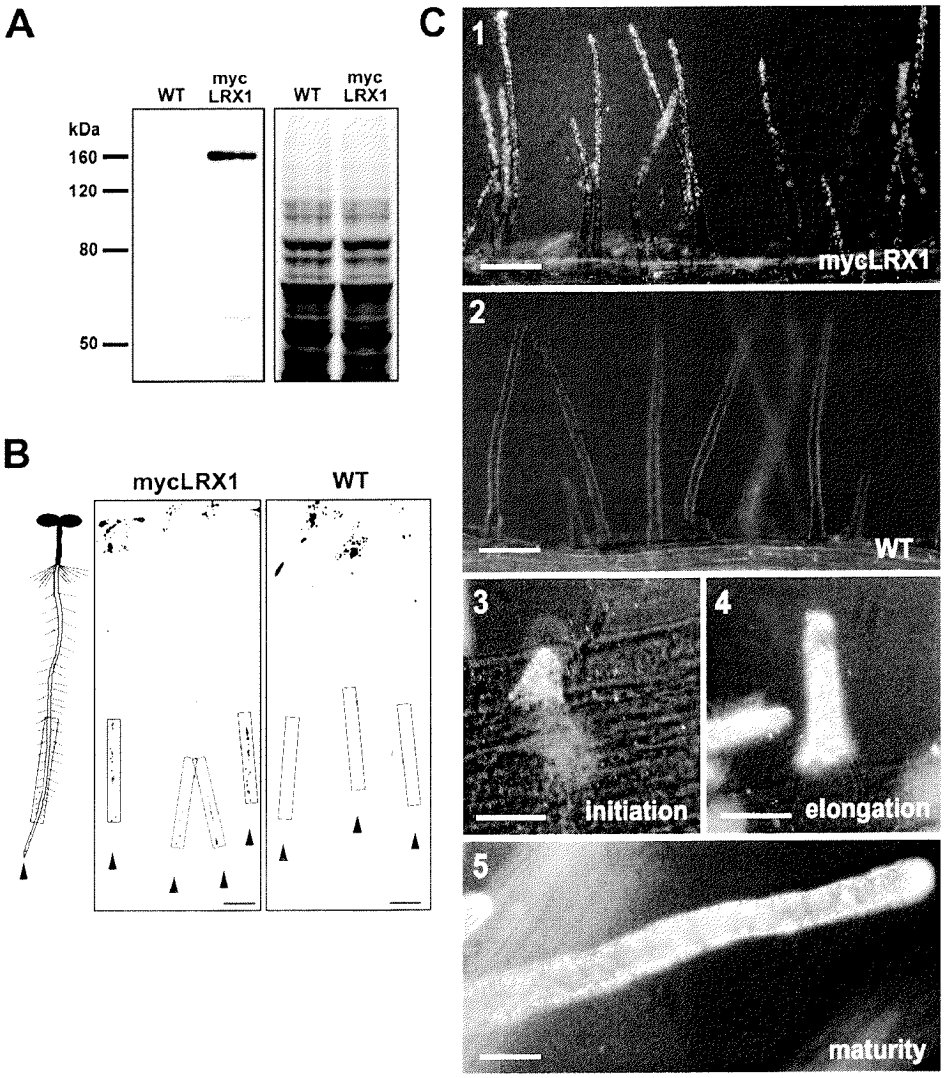


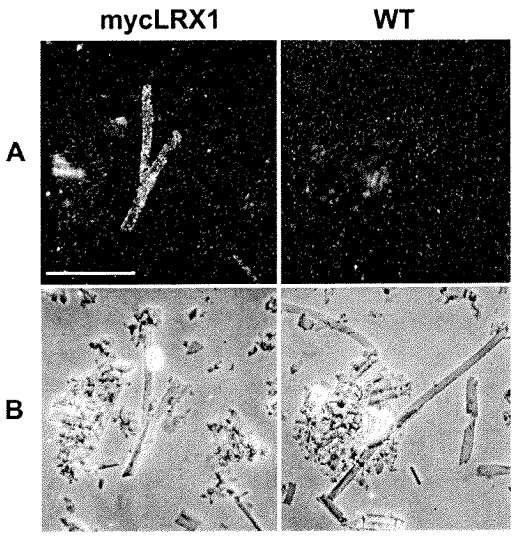


Figure 11. Immunodetection of mycLRX1 in purified cell walls. Wild-type (*WT*) and mycLRX1 (*mycLRX1*) roots were ground in liquid nitrogen and cell walls were purified before immunolabeling with myc-mAbs and gold-conjugated secondary antibodies. After silver enhancement, the preparation was observed under epipolarized light (*A*) to reveal the labeling, or transmitted light (*B*) to identify cell types. Tubular structures, identified as root hair debris were regularly labeled in mycLRX1 material, whereas such structures were not decorated in wild-type preparations. Bar, 100μm.

4.2.5 Identification and characterization of transposon-tagged lrx1 *mutants*

To investigate the biological function of LRX1, we identified *lrx1* loss-of-function mutants by screening an *En-1*-mutagenized *Arabidopsis* population. Three independent lines were identified which carried an insertion in the *LRX1* gene. These were named *lrx1-1*, *lrx1-2*, *lrx1-3*, according to the position of the insertion in the sequence (Fig. 5). The three mutant lines were backcrossed at least four times into wild-type background to reduce the number of additional *En-1* insertions, which initially ranged from 15 to 25 (Fig. 12A). The presence of the mutant *lrx1* allele was verified in each generation by RFLP analysis (Fig. 12B). In the line *lrx1-2*, all the additional insertions could be crossed-out, whereas a genetically closely linked insertion co-segregated with the insertion in the *LRX1* gene in the line *lrx1-3* (Fig. 12C). Following several back- and self-crosses aimed at reducing the number of irrelevant *En-1* insertions in the line *lrx1-1* (generations F_1, F_2 and F_3, Fig. 13), the progeny of a plant heterozygous for the presence of an *En-1* element at the *LRX1* locus was screened at the phenotypic level to isolate a homozygous mutant line (F_4, Fig. 13). Surprisingly, the progeny showed a homogenous *lrx1* mutant phenotype. PCR amplification and subsequent sequencing of the *LRX1* locus with primers flanking the *En-1* insertion point generated products corresponding to the wild-type allele sequence except for the presence, at the En-1 insertion point, of 4 additional nucleotides left behind by self-excision of the transposable element, presumably in the previous generation (Fig 13, *box*). Plants homozygous for this stable mutant allele were isolated in order to conserve a genetically stable *lrx1* mutant line (line *lrx1-1fp*). Southern analysis with an *En-1* probe showed that this line is free of any remaining *En-1* element (Fig. 12C).

Eventually, *LRX1* expression was tested in the three mutant lines by Northern hybridization. No transcript could be detected in the lines *lrx1-1* and *lrx1-3* whereas in the line *lrx1-2*, a shorter *LRX1* transcript of 1.3 kb was detected at a much lower level compared to wild type (Fig. 12D).

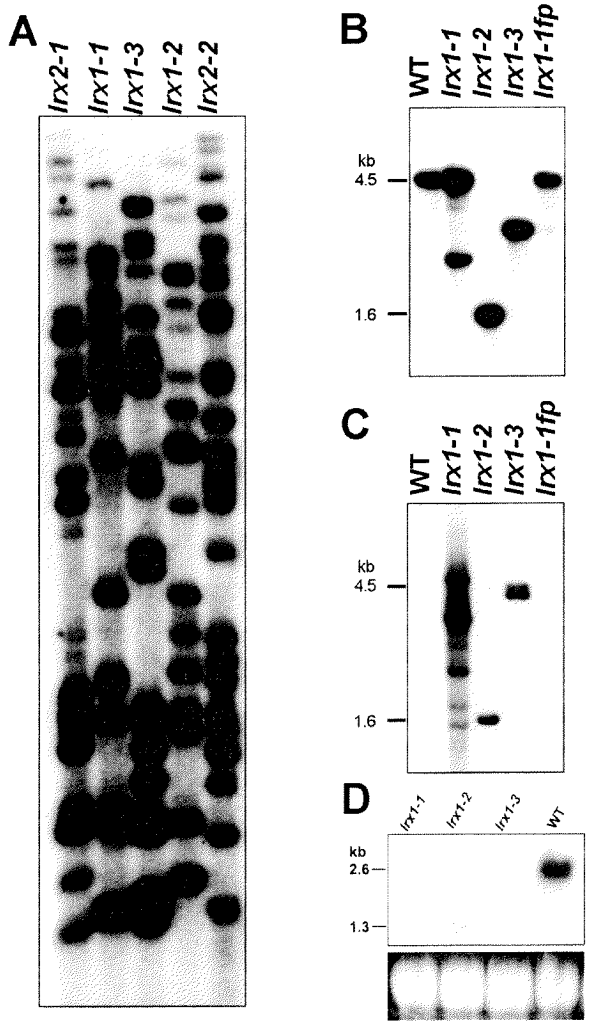


Figure 12. Isolation of transposon-tagged *lrx1* mutants. (*A*) Southern blot of *Eco*RV-digested genomic DNA of three lines identified to contain an *En-1* element in the *LRX1* gene and two lines with an insertion in the *LRX2* gene. The membrane was hybridized with a ^{32}P-labelled probe derived from the left border of the *En-1* element. (*B*) Southern blot of *Eco*RV-digested genomic DNA of wild-type and homozygous *lrx1* mutant lines. The membrane was hybridized with a ^{32}P-labelled LRX1 probe. The insertion of the *En-1* element causes specific RFLP patterns for each of the lines. The lane *lrx1-1fp* contains DNA of *lrx1-1* mutant plants where the transposon excised from the *LRX1* locus leaving a foot print. The RFLP pattern of those stable mutants is identical to that of wild-type plants. (*C*) same blot as (*B*) hybridized with the *En-1* specific probe. The *lrx1-2* and *lrx1-3* lines contain one and two *En-1* insertions, respectively, after four crosses with wild-type plants. In the *lrx1-1fp* line insertions have been completely out-crossed. The *lrx1-1* plants used for this blot had only been crossed twice with wild-type *Arabidopsis* and therefore still contain several *En-1* insertions. (*D*) *LRX1* expression in *lrx1* mutants. Total RNA was extracted from 14-day-old seedlings grown vertically on the surface of MS plates. Seedlings from wild-type Columbia (*WT*) and from each of the three *lrx1* mutant lines (*lrx1-1, -2, -3*) were used. Total RNA (20 μg) was used for Northern hybridization. 25S rRNA was used as loading control (*lower panel*).

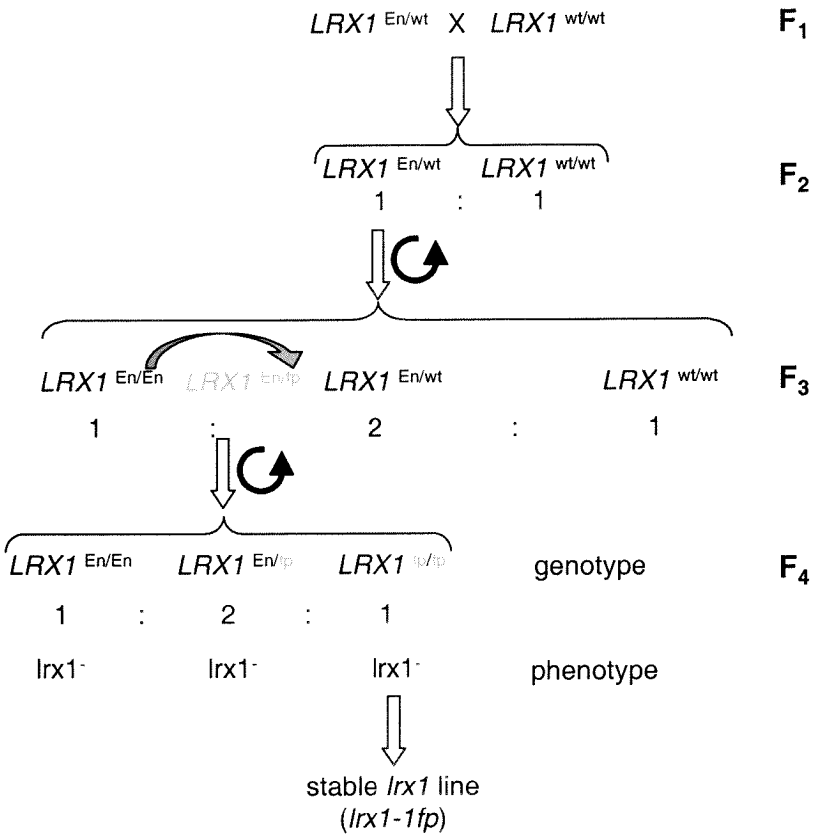


Figure 13. Identification of a stable *lrx1* mutant line. The scheme depicts the different *lrx1-1* generations, their genotype, and the events which led to the isolation by chance of a genetically stable *lrx1-1* line due to the presence of a footprint left by transposon excision. Generations F_1 to F_3 were analyzed by Southern hybridization to identify plants containing a reduced number of irrelevant *En-1* insertion. In the generation F_3 , a plant selected for its clear genetic background and determined, by RFLP analysis, as heterozygous for the *En-1* element at the *LRX1* locus, was selfed and its progeny tested (F_4). Hundred percent of the progeny displayed the *lrx1* mutant phenotype (lrx1$^-$). Sequencing of the PCR amplified *LRX1* locus revealed the presence of a foot print. Southern analysis identified plants homozygous for the *lrx1* allele carrying the foot print (*LRX1* $^{fp/fp}$). The lower box encloses the LRX1 sequence at the *En-1* insertion point for the wild-type allele (LRX1^{wt}), the *En-1* disrupted allele (LRX1^{En}), and the allele harbouring a foot print (LRX1^{fp})

Root hair development was compared between wild-type and homozygous mutant plants grown vertically on the surface of agar plates. In contrast to wild-type plants which showed an even pattern of straight and thin root hairs (Fig. 14A, C, and E and Fig. 15A), *lrx1* mutants exhibited an extremely irregular root hair development (Fig. 14B, D, F). The majority of the root hairs did not elongate completely and was shorter at maturity than wild-type root hairs (Fig. 14A-B and Fig. 15D-I). *lrx1* root hair development was often arrested early after initiation resulting in short stumps (Fig. 15H). Root hairs which continued to grow, frequently branched with one or two lateral branches emerging from the central stalk (Fig. 15D, F, G). *lrx1* root hairs frequently showed swelling, either at the basis (Fig. 15D, G) or along the main stalk (Fig. 15E, I), resulting in root hairs of irregular diameter or, in some extreme cases, in a spherical structure several fold the normal diameter of a root hair (Fig. 14F). Root hair statistics indicate that although the same number of root hairs initiate in *lrx1* mutants compared to wild type, a large proportion of them do not elongate further than 40 μm, when tip growth actually starts. The occurrence of other defects was quantified: 5.3% of the root hairs showed multiple branches, most frequently two, and 18.7% underwent swelling (Table 1). A two-fold local increase of the root hair diameter compared to the rest of the hair was considered as "swelling". Smaller irregularities were not quantified. The length of the longest root hairs in the *lrx1* mutant was significantly shorter than the average length of the wild-type root hairs. This indicates that even when the *lrx1* root hairs successfully establish tip growth and elongate, their size at maturity remains negatively affected, possibly as a consequence of a reduced elongation rate. Time lapse observations also revealed that root hairs often burst out and collapse, suggesting a sudden cell wall rupture due to a decreased resistance of the cell wall to the cell osmotic pressure (Fig. 16).

Trichoblast morphology, except for the hair itself, was not affected and the basal location of the root hair initiation site (Dolan et al. 1994) was preserved (data not shown). Root hair differentiation also took place at the same distance from the root tip in the mutants compared to the wild type, and a similar number of root hairs was initiated (Fig. 14C-D and Table 1). Furthermore, the alternate pattern of trichoblast and atrichoblast cell files as well as the inner anatomical organization of the root was indistinguishable between mutant and wild-type

roots (Fig. 17). The three mutant lines displayed the same phenotype and can be considered as null mutants, considering the absence of *LRX1* transcript in two of the lines. *lrx1* mutants grew normally on soil and did not display any defect besides altered root hair development. The mutant phenotype segregated in a 1:3 ratio in the progeny of a *lrx1* heterozygous plant, indicating that the *lrx1* mutation is recessive.

Table1. Root hair statistics in wild type and lrx1 mutants

	Hair density[a] (#/mm of root)	Hairs > 40μm[b] (%)	Hair length[c] (μm)	Branched hairs[b] (%)	Swollen hairs[b] (%)
Wild type	37.5 ± 2.5 (30)	100 (20)	635 ± 87 (50)	0.17 ± 0.65 (30)	0 (30)
lrx1	35.6 ± 2.5 (30)	57.9 ± 8.6 (20)	355 ± 94 (50)	5.3 ± 4 (30)	18.7 ± 7.2 (30)

[a] The hair density was measured on 1 mm of the mature zone of each root.
[b] This percentage refers to the same region used for the hair density measurement.
[c] In wild-type roots the value is the average of 5 hairs randomly chosen per root; for the *lrx1* mutants the five longest hairs of each root were measured instead.
The numbers between parenthesis indicate the sampling size.

A complementation test was performed by crossing a transgenic plant containing the mycLRX1 construct with a *lrx1* mutant. Analysis of the F_2 and F_3 progeny revealed that the mycLRX1 construct fully rescued the mutant phenotype (data not shown). This result demonstrates that the *lrx1* mutation is responsible for the observed root hair phenotype. It also shows that the mycLRX1 transgene is correctly expressed and encodes a fully functional protein, which is localized in the correct subcellular compartment.

A complementation test with previously isolated *rhd1, 2, 3,* and *4* root hair development mutants (Schiefelbein and Somerville 1990) was also performed. The analysis of the F_1 seedlings of the corresponding crosses revealed that *lrx1* is not allelic to any of the *rhd* mutants. It was also excluded that *lrx1* is allelic to the other described mutants affecting root hair morphogenesis (*tip1*, Schiefelbein et al. 1993; *cow1*, Grierson et al. 1997; *shv1* to *shv3*, *cen1* to *cen3*, *bst1* and *scn1*, Parker et al. 2000) by comparing the position of the loci on the genetic map. Therefore, *LRX1* constitutes a new gene involved in root hair development.

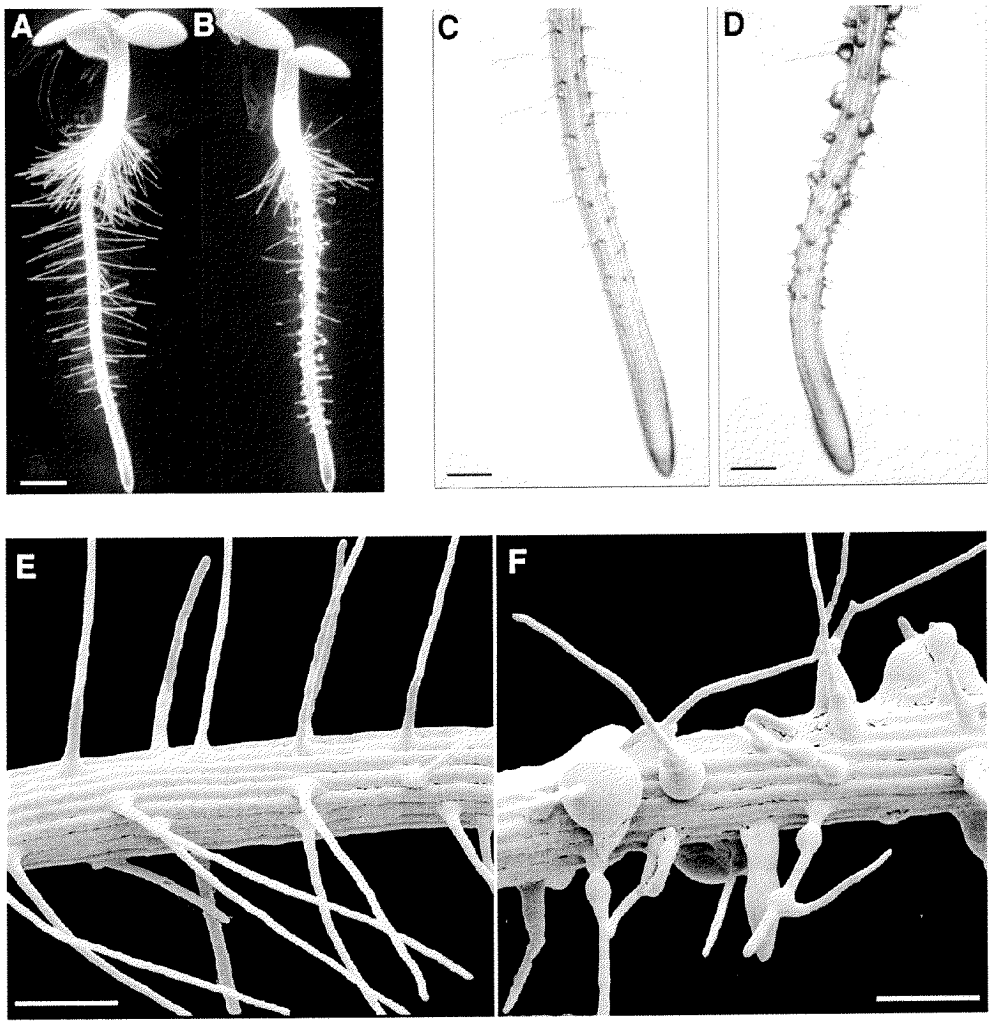


Figure 14. *lrx1-1* mutant phenotype. Wild-type and *lrx1-1* mutant seedlings were grown vertically for three days on the surface of MS plates and were either observed under a stereomicroscope with dark field illumination (*A, B*), transmitted light (*C, D*) or frozen in liquid nitrogen and investigated at low temperature in a scanning electron microscope (*E, F*). Compared to wild type (*A, C, E*), the *lrx1-1* mutant (B, D, F) has fewer fully elongated root hairs (*B*), and mutant root hairs frequently have a swollen basis (*D, F*), show an irregular diameter and often branch (*F*). The three *lrx1* mutant lines had identical phenotypes. Bar, 1mm (*A, B*), 350 μm (*C, D*), 250 μm (*E, F*).

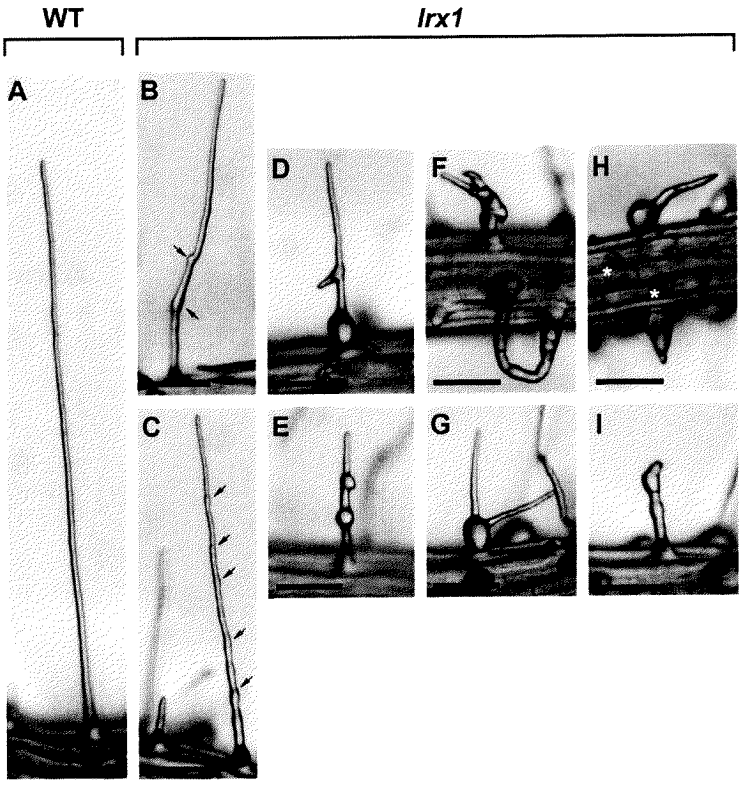


Figure 15. Details of the diverse root hair phenotypes observed in the *lrx1* mutants. *lrx1* root hairs (*B-I*) are shorter than wild-type root hairs (*A*) have irregular diameter (*B,C,* arrowheads), swell locally (*D, E, H, I*) and branch (*D, F, G*). Bar, 100 μm.

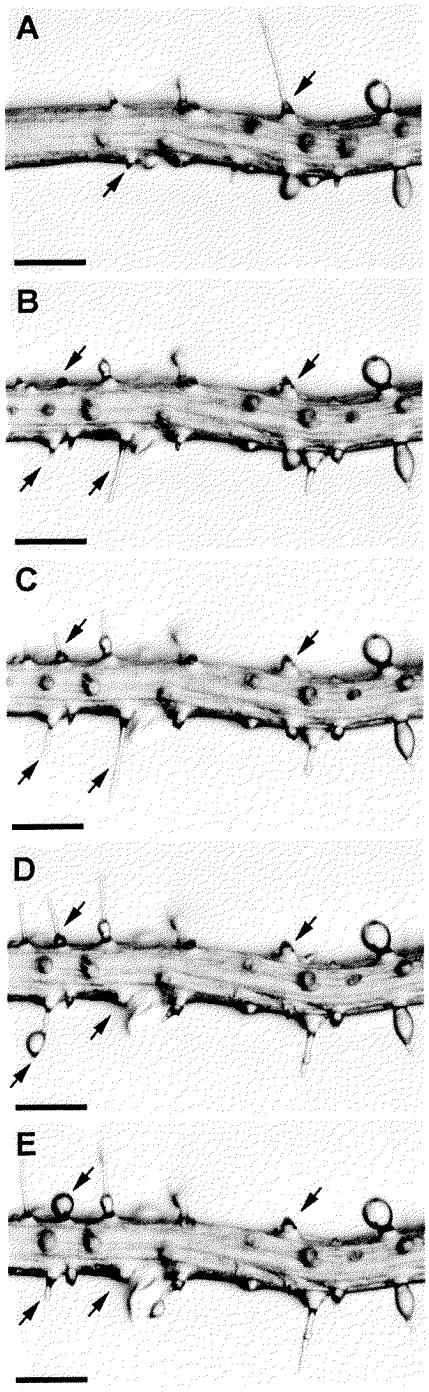


Figure 16. Time lapse observation of *lrx1* root hair growth. The same portion of the differentiation zone of a 3 day-old seedling is represented at 40 min intervals. Arrows indicate the same root hairs on different panels. The indicated root hairs collapsed during growth. Bar, 200 μm.

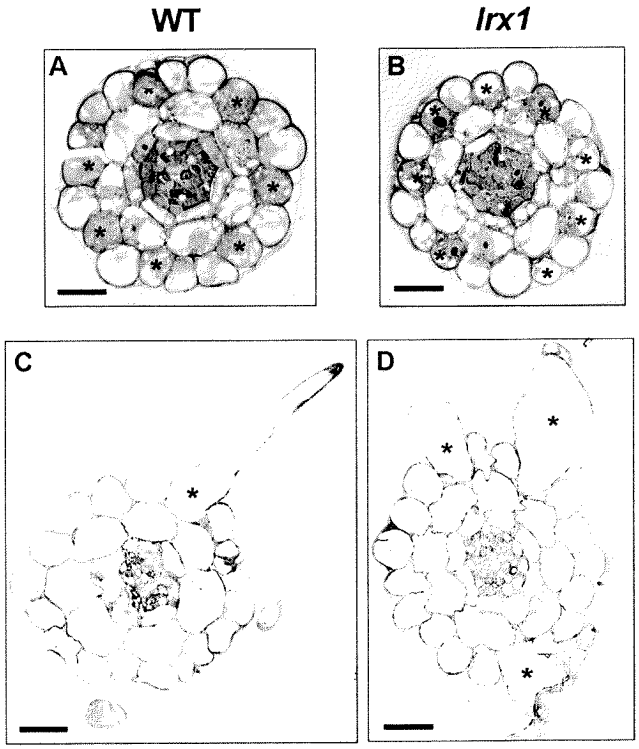
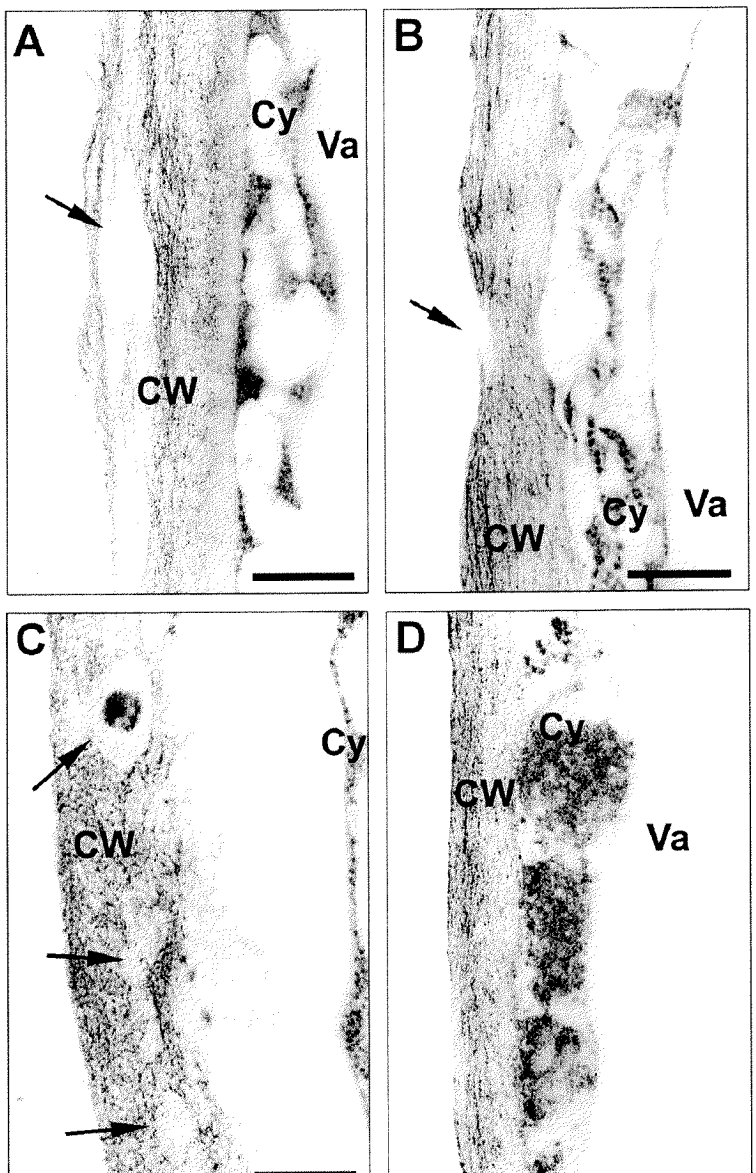


Figure 17. Anatomy of wild-type and *lrx1* roots. (*A-B*) Sections were made at the transition between the meristem and the elongation zone. The cells which, according to their position, will differentiate into trichoblasts are indicated with a star. They usually have a denser cytoplasm. (*C-D*) Sections were made in the differentiation zone. Stars indicate trichoblast cells cut at the level of the hair proper. (*A*, *C*) Wild type, (*B*, *D*) *lrx1* mutants. Bar, 25 μm).

Figure 18. Ultrastructure of the root hair cell wall in wild type and *lrx1* mutants. The pictures were taken close to the basis of mutant (*A*, *B*, *C*) and wild-type (*D*) root hairs, respectively. (*A*) The outer cell wall layer is separated from the rest of the wall. (*B*) The cell wall shows local thinning. The outer layer is apparently disrupted. (*C*) Unknown non-fibrillar inclusion and low electron density areas are visible in the wall. The large region between the cell wall and the cytoplasm is likely to be a fixation artefact. The arrows point to the above mentioned defects, (*CW*) cell wall, (*Cy*) cytoplasm, (*Va*) vacuole. Bar, 0.25 μm.

4.2.6 The cell wall ultrastructure in the lrx1 *mutants shows localized defects*

Thin sections of similar regions of both wild-type and mutant root hair were observed with an electron microscope. Although no regular difference was observed in the cell wall of the *lrx1* mutant root hairs compared to the wild type, in several places the cell wall showed localized and discrete aberrations such as disintegration of the outer layer (Fig. 18A), reduced thickness (Fig. 18B), inclusions of unknown material (Fig. 18C) and zones of low electron density (Fig. 18C). In several cases, the defects affected only the outer part of the cell wall while the inner part appeared normal. Since in these preliminary investigations no statistical or quantitative measurements were performed, it is possible that there are also more subtle differences which distinguish the cell walls of wild-type and mutant root hairs, respectively.



4.2.7 Genetic interactions between lrx1 *and* rhd *mutants*

Double mutants *lrx1rhd1*, *lrx1rhd2*, *lrx1rhd3*, *lrx1rhd4* were generated to determine more precisely the role of *LRX1* in the root hair developmental pathway. Comparison of the double mutant phenotype with the phenotype of both parents indicated additive effects for *lrx1rhd1*, *lrx1rhd3* and *lrx1rhd4* and a clear epistatic effect of *RHD2* on *LRX1*, since the *lrx1rhd2* phenotype is identical to the *rhd2* mutant. In each case of additive effects, the characteristics of the rhd parent were apparent but the number of root hairs reaching maturity was reduced, as observed in *lrx1* (Fig. 19A). As *lrx1* and *rhd4* have very similar phenotypes under our conditions, the phenotype of the *lrx1rhd4* is less easy to distinguish from the parental phenotypes. However, the root hair defects, though qualitatively similar, appear quantitatively more important in the double mutant.

The expression of *LRX1* in the *rhd1*, *rhd2*, *rhd3* and *rhd4* mutant backgrounds was verified by Northern hybridization. The level of the *LRX1* transcript was much reduced in *rhd1*, *rhd2*, and *rhd4*, whereas it was only slightly lower in *rhd3* background (Fig. 19B). This reduction in the expression of *LRX1* is correlated with the severity of the defect affecting root hair development in the rhd mutants. *rhd3* root hairs are waving and shorter than wild-type root hairs but their number is not reduced, whereas in *rhd1* and *rhd4* fewer root hairs elongate further after initiation. Root hair growth is almost completely prevented in *rhd2*. These results confirm the previous data obtained with AVG treated and *rhd6* mutants (Fig. 7E) and indicate that *LRX1* expression is dependent on the proper root hair elongation. In addition these data suggest the existence of a feed-back regulation, since *LRX1* expression is reduced in cases where elongation of the root hairs is disturbed but not their viability, as in *rhd3*.

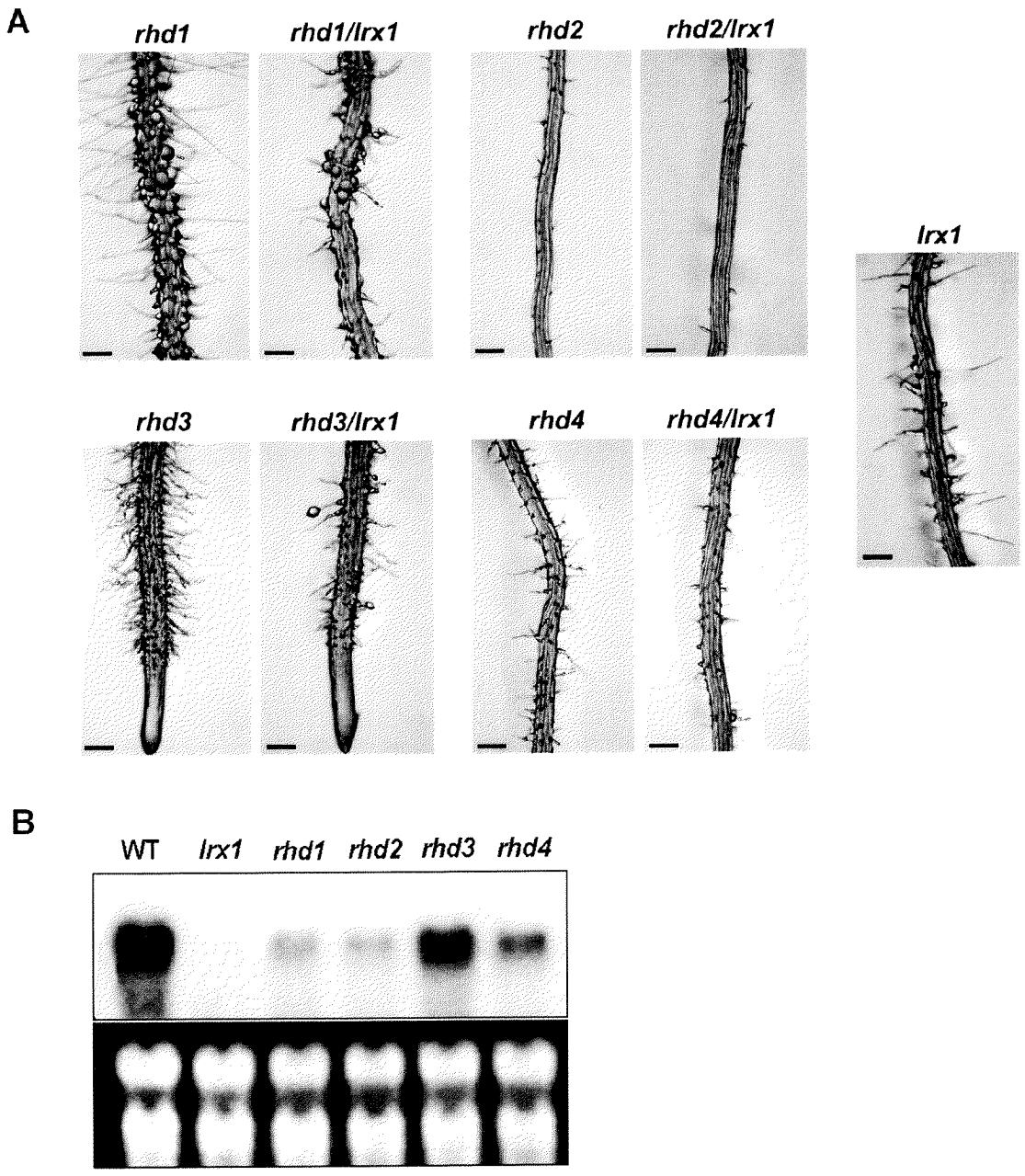


Figure 19. Genetic interaction of the *lrx1* mutation with other root hair mutations. (A) Phenotype of double mutants generated between *lrx1* and *rhd1, rhd2, rhd3* and *rhd4* root hair mutants. For comparison, a picture of each of the single mutant parents is shown. *rhd1/lrx1*, *rhd3/lrx1* and *rhd4/lrx1* have a stronger phenotype than the respective single mutants. *rhd2/lrx1* is identical to *rhd2*. (*B*) *LRX1* expression in different mutant background. *LRX1* expression is strongly reduced in the *rhd1*, *rhd2* and *rhd4* mutants but only slightly in the *rhd3* background. The lower panel shows ribosomal RNAs as a loading control. Bar, 200 μm.

4.2.8 Constitutive overexpression of full-length and truncated LRX1 proteins

The *LRX1* gene under the control of the 35S CaMV promoter was transformed into wild-type *Arabidopsis*. Several transgenic lines displayed an *LRX1* transcript level much higher than wild-type plants and one representative line was selected for further analysis. The higher *LRX1* expression in the roots of the 35S-LRX1 transgenic plants compared to wild type was demonstrated by Northern hybridization and protein immunoblotting (Fig. 20A and B). For immunodetection, the anti-LRX1 polyclonal antiserum raised against the LRR domain of LRX1 was used. The phenotype of the 35S-LRX1 plants was analyzed on MS plates and on soil. No difference in growth or morphology could be detected between transgenic and wild-type plants, indicating that an excess of LRX1 does not affect plant development (Fig. 20C, panel 2). Complementation of the *lrx1* mutant by the 35S-LRX1 construct confirmed that the overexpressed LRX1 is functional (data not shown).

To study the role of the LRR domain, transgenic plants were generated that expressed, under the control of the 35S CaMV promoter, a modified LRX1 protein whose extensin domain had been deleted (35S-N/LRX1). Protein immunoblotting revealed high expression of a polypeptide with an apparent molecular mass of 45 kDa in 15 out of 18 transgenic lines (data not shown). The size of the detected protein is in good agreement with the calculated mass of the truncated LRX1 protein (35 kDa). All the lines expressing the N/LRX1 protein displayed a strong defect in root hair development, which completely phenocopied the *lrx1* mutants (Fig. 20C, panel 3). No other alteration in development was observed in these transgenics. One representative homozygous transgenic line harboring a single T-DNA insertion and showing a high N/LRX1 expression was further characterized by Western and Northern analysis (Fig. 20A and B,2). Northern hybridization demonstrated that the expression of the endogenous *LRX1* gene was not reduced in 35S-N/LRX1 plants (Fig. 20A, 2). Hence, the observed phenotype is not caused by silencing of the endogenous *LRX1* gene but is the result of a dominant negative effect due to the overexpression of the truncated LRX1 protein.

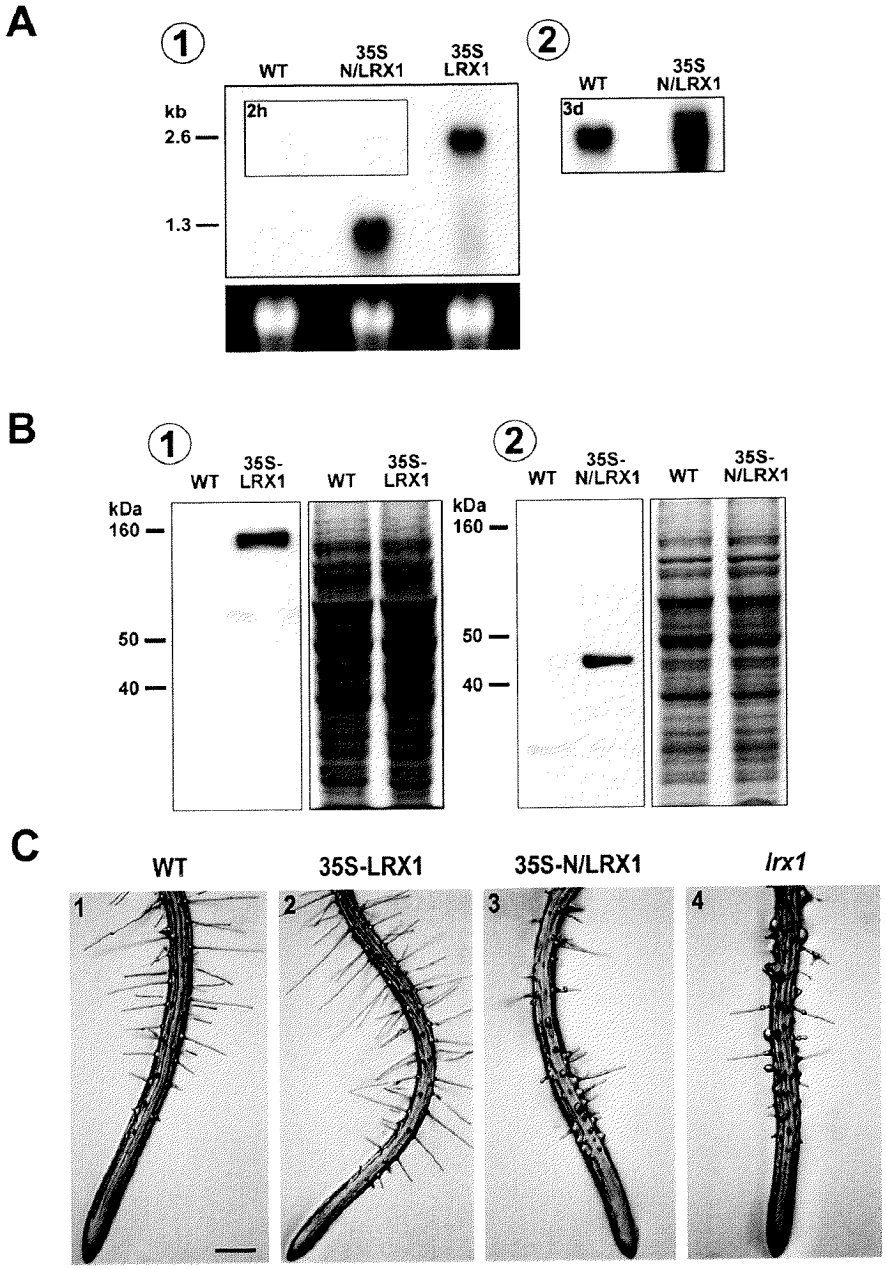


Figure 20. Overexpression of the complete (35S-LRX1) and truncated (35S-N/LRX1) LRX1 protein. The sequences coding for either the full-length or the truncated LRX1 protein were transformed into *Arabidopsis* under the control of the 35S CaMV promoter. (*A*) Total RNA was extracted from roots of 14-day-old wild-type seedlings (*WT*), transgenic seedlings overexpressing the truncated LRX1 (*35S-N/LRX1*) and transgenic seedlings overexpressing the complete LRX1 (*35S-LRX1*). Total RNA (5 μg) was used for Northern hybridization with a ^{32}P-labeled LRX1 probe (Fig. 1A). The membrane was exposed first for 2 hours (*1*) and the *framed* area in (*1*) was further exposed for three days (*2*). Transgene transcript levels were more than hundred-fold higher than the level of the endogenous *LRX1* transcripts. The lower panel shows 25S ribosomal RNA as a loading control. (*B*) Immunoblot of root protein extracts from wild-type and 35S-LRX1 plants (*1*) and wild-type and 35S-N/LRX1 plants (*2*). The membrane was immunoreacted with anti-LRX1 antiserum. The endogenous LRX1 protein was not detectable with the anti-LRX1 antibodies under the conditions used for these blots. A duplicate gel was stained with Coomassie blue as loading control (*right panels*). (*C*) Phenotype of the lines used in (*A*) and (*B*). 35S-LRX1 plants (*2*) are indistinguishable from wild-type plants (*1*) while 35S-N/LRX1 plants (*3*) show the same phenotype as *lrx1* mutants (*4*). Bar, 350 μm.

4.3 Molecular characterization of LRX2

4.3.1 Genomic sequence analysis

The study of the second LRX gene detected in the *Arabidopsis* genome (*LRX2*) was initiated following the same approach as used for *LRX1.* However, as the project developed, investigations focused mainly on *LRX1* and our results are by far not as complete for *LRX2.*

The *LRX2* gene is located on chromosome IV, BAC clone F24O1, and consists of an intronless open reading frame of 2358 bp encoding a protein of 786 amino acids. The full genomic sequence of *LRX2* was isolated by screening a λ ZAPII genomic library with the *LRX1* probe since *LRX1* and *LRX2* are 82% homologous in their LRR DNA sequence. A clone containing the open reading frame and 1.55 kb of 5' promoter sequence as well as 1.63 kb of the 3' sequence was identified.

The predicted LRX2 protein contains, as every member of the LRR/extensin family, a signal peptide, an LRR domain and an extensin domain. Its LRR domain is 261 amino acids long and shares 85% of identity with the LRR domain of LRX1, which makes LRX2 the LRX protein most closely related to LRX1 (Fig. 21). Identity with TOML-4 and PEX1 is 75% and 58%, respectively. The extensin domain is organized in sub-domains of repeated sequences which are similar to the repeats found in LRX1. A first block of six P_4S_2KMSPSFRAT strongly ressembles the four repeats SP_5S_2KMSPSVRAY found in LRX1 (Fig. 21 and Fig. 5). The *LRX2* sequence was found only once in the *Arabidopsis* genome sequence and Southern blot analysis confirmed that it is a single copy gene (Fig. 22).

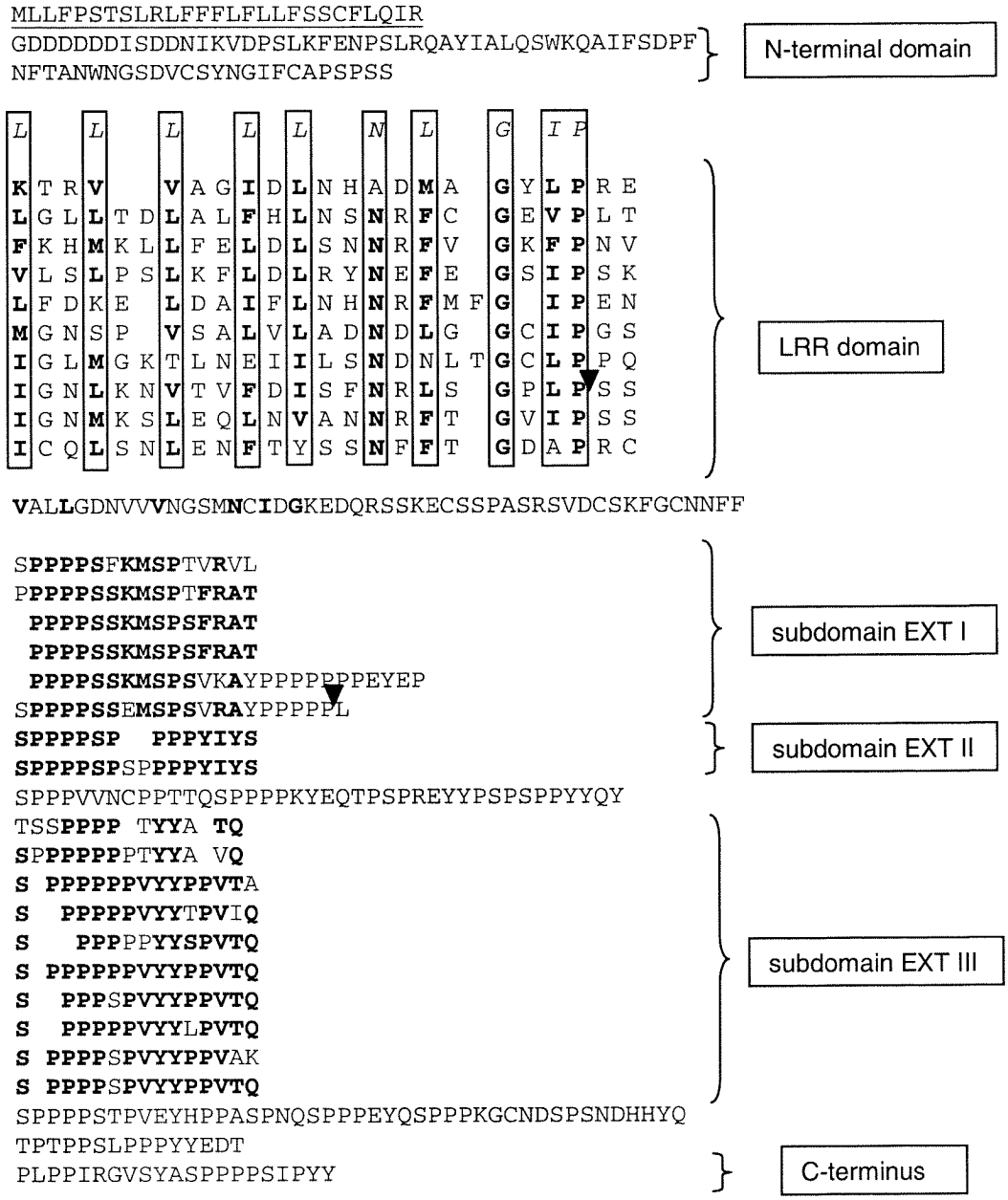


Figure 21. Deduced amino acid sequence of the *LRX2* gene. The predicted signal peptide is underlined and the different domains have been arranged to highlight their organization. The LRRs are aligned with the plant extracellular LRR consensus sequence (*first line in the frames*). The conserved amino acids are indicated in bold. The diverse higher-order repeats of the extensin domain are aligned and the amino acids conserved within those repeats are written in bold. Black arrowheads indicate the position of the two *En-1* insertions.

4.3.2 LRX2 *is predominantly expressed in roots*

The expression pattern of *LRX2* was determined by Northern hybridization, RT-PCR, and analysis of transgenic plants harbouring the *uidA* gene under the control of the *LRX2* promoter (*pLRX2::GUS*). Northern blot (Fig. 23A) and RT-PCR (Fig. 23B) experiments revealed that *LRX2* is mainly expressed in roots and very weakly in leaves. The size of the hybridizing transcripts was in agreement with the prediction from the *LRX2* sequence. The root specific expression was also confirmed by GUS activity in the root meristematic region and in the rhizodermal cells along the differentiation zone (Fig. 23C). In some cases, GUS staining was also observed in the inner cell layers, particularly the cortex. However, the resolution of whole mount observations was not sufficient to determine if this reflects the true expression of the *pLRX2::GUS* construct or results from the diffusion of the reaction product. Meristematic expression was observed very shortly after emergence of the primary root and was similarly observed in lateral roots where the same pattern was reiterated (Fig. 23C, 1). A very faint and diffuse staining was observed in mesophyll cells of the leaves and in the hypocotyl of the young seedlings (Fig. 23C, 2).

4.3.3 Identification of transposon-tagged lrx2 *mutants*

Two *lrx2* mutant lines were identified by screening with *LRX2* gene specific primers the same *En-1* mutagenized population which had been used to isolate the *lrx1* mutants. In the first line (*lrx2-1*) the gene is disrupted by the *En-1* element inserted at the end of the LRR domain whereas in line *lrx2-2* the transposon is located at the beginning of the extensin domain (Fig. 21). The plants were backcrossed into wild-type background to reduce the number of additional insertions (data not shown). The analysis of a population segregating for the mutant allele did not reveal any morphological defect in any of the plants and growth in soil was normal. Further investigations of plants grown in different conditions as well as anatomical studies will be required to confirm the absence of a phenotypical change.

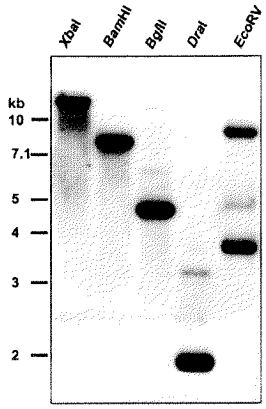


Figure 22. Southern blot of *Arabidopsis* genomic DNA hybridized with a *LRX2* specific probe. Genomic DNA digested with the restriction enzymes indicated at the top was hybridized with a ^{32}P-labelled *LRX2* probe.

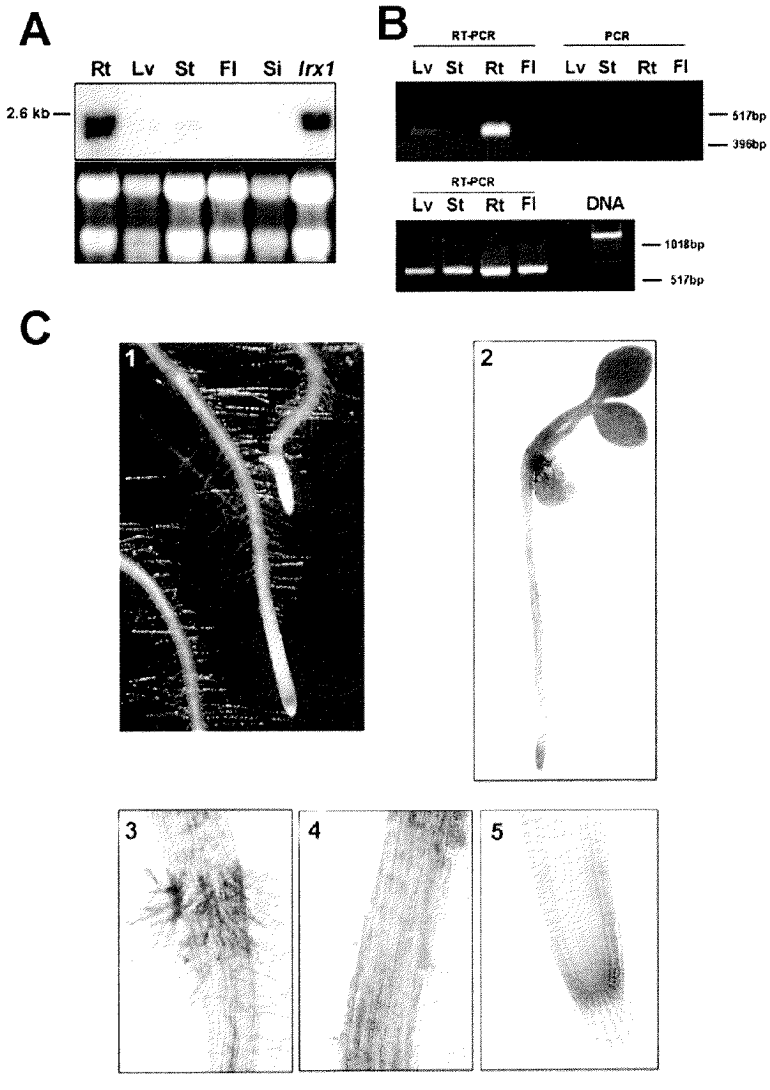


Figure 23. *LRX2* expression pattern. (*A*) Organ-specific expression of *LRX2*. Total RNA was extracted from green siliques (*Si*), flowers and flower buds (*Fl*), inflorescence stems (*St*), and rosette leaves (*Lv*), of 35 to 40-day-old wild-type Columbia plants. Roots (*Rt*) were harvested from 14-day-old seedlings grown vertically on MS medium. For Northern analysis, total RNA (5 µg) was hybridized with a ^{32}P-labeled *LRX2* probe and 25S rRNA was used as loading control (*lower panel*). RNA from *Irx1* mutants was used to control that signals are not the result of cross-hybridization with *LRX1* trancripts. (*B*) RT-PCR experiments with the same RNA as in (*A*). *LRX2* transcripts were amplified by PCR with gene specific primers after reverse transcription (see legends of fig. 7). (*C*) Transgenic seedlings containing the *LRX2* promoter fused to the *uidA* gene were histochemically stained to reveal the tissue-specific expression of *LRX2*. GUS activity (blue staining) was observed in the meristematic region of the root (*1, 2, 5*), along the differentiation zone (*1, 2, 4*) and in the root hairs of the collet region (*2, 3*). Some staining was also observed in the hypocotyl (*2*).

4.3.4 Expression of the LRR domain in E. coli *and production of anti-LRX2 antibodies*

The *LRX2* sequence encoding the LRR domain was amplified by PCR and cloned as a translational fusion with a 6x His tag into the expression vector pQE30 (recLRX2). The construct was transformed into *E.coli* and the expression of the recombinant LRX2-LRR domain was monitored by SDS-PAGE of the crude bacterial protein extract. A prominent band at 28 kDa confirmed the expression of the recombinant polypeptide by the bacteria (data not shown). RecLRX2 was purified by metal chelate affinity chromatography on Ni-NTA agarose beads and purity of the preparation was verified by SDS-PAGE (data not shown). The denaturated protein diluted in 8M urea was used to immunize two rabbits. The IgG fraction was purified on a protein A affinity column and used as such in subsequent experiments. The quality of the antiserum was tested on a crude bacterial protein extract from the strain expressing recLRX2 (data not shown).

4.3.5 Constitutive overexpression of full-length and truncated LRX2 proteins

The *LRX2* gene under the control of the 35S CaMV promoter was transformed into wild-type *Arabidopsis*. Several independent T_2 progenies were grown at the surface of the medium and screened for a mutant phenotype. No difference between the transgenics and the wild-type plants was observed. However, preliminary protein immunoblotting with anti-LRX2 polyclonal antibodies failed to detect the recombinant LRX2 protein. The expression of the transgene needs to be assessed by Northern hybridization. Plants expressing a modified LRX2 protein missing the extensin domain under the control of the 35S CaMV promoter were also produced (35S-N/LRX2). The expression of the transgene was tested by protein immunoblotting on T_2 progeny and several lines showed the presence of a 45 kDa polypeptide absent in wild-type protein extracts (Fig. 24). The transgenic lines expressing N/LRX2 were grown vertically on the surface of MS medium and were examined for the presence of a mutant phenotype. No difference was observed between 35S-N/LRX2 plants

and wild-type plants, demonstrating that overexpression of the truncated LRX2 protein does not affect plant growth and development (data not shown).

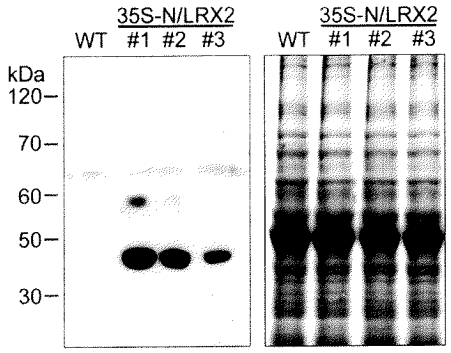


Figure 24. Overexpression of a truncated (35S-N/LRX2) LRX2 protein. The sequences coding for the truncated LRX2 protein were transformed into *Arabidopsis* under the control of the 35S CaMV promoter and protein extracted from three T_2 lines were immunoblotted. The membrane was immunoreacted with anti-LRX2 antiserum. 35S-N/LRX2 plants express a 40 kDa polypeptide which is absent from the wild-type untransfromed control. The endogenous LRX2 protein was not detectable with these antibodies. A duplicate gel was stained with Coomassie blue as loading control (*right panel*).

5. Discussion

In this study, the existence of genes encoding chimeric LRR/extensin proteins was confirmed. These genes constitute a large family in *Arabidopsis* and occur in other distantly related species such as tomato and maize. The function of *LRX1*, the first *Arabidopsis* LRR-extensin gene identified, was investigated and the results presented here indicate that the encoded protein is an important component of root hair morphogenesis.

5.1 LRR/extensins form a novel sub-family of LRR-proteins

All the *LRX* genes identified by their similarity with the LRR sequence of *TOML-4* encode proteins which share essentially the same chimeric structure: a carboxy-terminal extensin-like domain and a domain matching the consensus sequence of plant extracellular LRRs. The latter have been found in proteins involved in signaling, such as receptor-like kinases, and in pathogenesis-related proteins like the polygalacturonase inhibitor proteins (Toubart et al. 1992). In receptor-like kinases, the LRRs constitute the extracellular ligand-binding domain of transmembrane proteins which, upon interaction with the ligand, undergo conformational changes or dimerization. These modifications are thought to activate the cytoplasmic kinase domain and trigger a signaling cascade (Torii 2000). In most cases, the nature of the ligand of plant LRRs is unknown although there is strong biochemical and genetic evidence indicating that the ligand of the receptor-like kinase CLAVATA1 (Clark et al. 1997) is the secreted peptide CLAVATA3 (Fletcher et al. 1999; Trotochaud et al. 2000). The identity of the ligand for PGIPs is also known since they interact with the fungal endopolygalacturonases and inhibit their enzymatic activity *in vitro* (De Lorenzo and Cervone 1997). In view of the similarity between the LRR domains of the LRX proteins and of CLAVATA1 or PGIPs, and considering that in other eukaryotes LRRs commonly mediate protein-protein interactions, one can postulate that LRX-LRR domains also interact with other polypeptides. Although the ten repeats of the LRR domain of the LRX family relate LRR/extensins to PGIPs, which also have a conserved number of ten LRRs, this resemblance is

superficial since, at the sequence level, the similarity with PGIPs is not higher than with other plant LRRs. It is therefore unlikely that the similar number of LRR reflects any common biological activity. Indeed, the sequence identity between the LRR domain of the LRX proteins and other LRR-proteins such as LRR-RLKs or PGIPs does not exceed 35-37%, whereas identity within the LRX proteins ranges from 55% to 95%.

5.2 Does the conservation of the LRR domain in the LRX family imply interaction with similar ligands ?

It might be tempting to postulate that the strong identity shared by the LRR domains of some LRX genes (95% between LRX6 and LRX11, 85% between LRX1 and LRX2) indicates recognition of identical or very similar ligands. However, *Cf-9* and *Cf-4* , two resistance genes encoding membrane proteins with extracellular LRRs, share 91% identity at the amino acids level, a difference which is apparently sufficient to confer resistance to different strains of the pathogenic fungus *Cladosporium* (Thomas et al. 1998). Domain swapping and gene shuffling experiments have furthermore demonstrated that only a fraction of the variant residues are actually responsible for the difference of specificity (Wulff et al. 2001). It was also shown that the specificity of Cf-4 and Cf-9 for their respective ligand is determined by residues at different positions. Most variation between LRR resistance genes and between PGIPs are confined within the xxLxLxx motif of the LRRs and evidence suggests that these motifs have been subjected to diversifying selection (Parniske et al. 1997; Ellis and Jones 1998). Crystallography studies of the ribonuclease inhibitors have shown that the xxLxLxx motif determines a β-strand/β-turn structure in which the amino acids flanking the Leu residues are solvent exposed and interact with the ligand (Kobe and Deisenhofer 1996; Papageorgiou et al. 1997). Recently, on the basis of protein structure modeling of a PGIP, a similar structure for plant LRRs has been proposed and site-directed mutagenesis has demonstrated that a single substitution in the β-strand/β-turn region confers a new specificity to the protein (Leckie et al. 1999). In the LRX family, the distribution of the variability appears to be different since there are less non conservative amino acid substitutions in the solvent exposed region than in the average sequence, suggesting that a

counter selection has been operating. While in the case of resistance genes and defense related genes sequence diversification is crucial to keep away rapidly evolving pathogens, mutations in genes involved in development are more likely to disrupt the function of the gene and therefore to be detrimental.

5.3 The LRX *family arose by duplication of an ancient set of genes*

The distribution of most of the *LRX* genes on duplicated chromosome fragments (Vision et al. 2000) suggests that the actual family arose from an ancient, reduced set of *LRX* genes. Duplication of the initial *LRX* genes has probably allowed one copy to evolve new specificities and possibly new functions. The present average of 10.4% of amino acid substitution in the β-strand/β-turn structure enables a wide range of specificities. The common origin of the paralogous *LRX* genes is still apparent in the higher sequence identity of their LRR domains but also of other parts of the protein, such as the C-terminus. In contrast, the apparent divergence of most of the extensin domains might be the consequence of their highly repetitive structure which could facilitate internal rearrangements. Thus, conservation of the basic motifs, particularly the sequence $SerPro_n$, might be sufficient to retain the overall extensin structure and possibly the function of the domain. In addition, the highly conserved C-terminus perfectly corresponds to the subgroups defined by the LRR domain similarity, independent of the differences in the extensin domains. This C-terminal sequence might also be crucial for protein function. E.g. the terminal Tyr residues might be involved in the cross-linking of the protein in the cell wall. Preliminary characterization of the expression pattern of the other members of the *LRX* family in *Arabidopsis* (*LRX3* to *LRX11*) has revealed that the different *LRX* genes can be classified into expression categories which almost perfectly overlap with the sequence similarity groups (data not shown). *LRX3*, *LRX4*, *LRX9* and *LRX10* are predominantly or exclusively expressed in flowers, and particularly in pollen, whereas *LRX5*, *LRX6*, and *LRX11* transcripts are detected in every organ. *LRX8* and *LRX7* have an intermediate pattern of expression with a strong expression in flowers and a low expression in the rest of the plant. Therefore, the pattern of expression of the *LRX* genes seems also to correspond to their ancient unique

origin and matches with the similarities of their sequences. It is difficult to determine if the correlation, within each LRX group, between the sequence homologies and the pattern of expression reflects conserved biological functions or merely represents the relic of a common origin. In the particular case of the group A, the overlapping but not identical pattern of expression of the paralogous *LRX1* and *LRX2* genes indicates that sequence diversification probably also occurred in the promoter region.

5.4 There is no functional redundancy between LRX1 and LRX2

Gene duplication frequently leads to redundancy, which explains why many knock-out mutants do not show a mutant phenotype. Functional redundancy within the different LRX groups is possible, especially in the group B which contains four genes expressed in pollen. In the case of *LRX1* and *LRX2,* the mutant phenotype shown by the *lrx1* mutants demonstrates that *LRX2* can not replace *LRX1*. Moreover, the observation that overexpression of the LRR domain of *LRX1* phenocopies the null *lrx1* mutant, whereas overexpression of the LRX2 LRR domain does not, strongly suggests that both LRR domains have different specificities and therefore can not be functionally exchanged. Another possible issue of gene duplication is the creation of pseudogenes. Since all the *LRX* genes were found to be expressed (data not shown), *LRX* family duplication does not appear to have generated pseudogenes.

5.5 Is the family now complete?

Eleven *LRX* genes have been identified in *Arabidopsis* after completion of the sequencing of its entire genome. This survey for *LRX* genes was performed by searching the databases with the LRR amino-acid sequence of *LRX1* and *LRX4* with the tBLASTn program. It can not be excluded that additional genes will still be discovered in the future since gene prediction errors or sequencing errors might have allowed *LRX* genes to escape detection. For instance, a close examination of the *LRX5* sequence revealed a probable sequencing error. The extensin domain was interrupted by a very short predicted intron whereas a shift of reading frame would allow the continuous translation of a characteristic

extensin sequence. Removal of a single nucleotide at position 2305 allows the formation of a unique open reading frame. The sequence attached in the appendix was corrected accordingly (the bases flanking the position of the nucleotide removed are indicated in bold).

5.6 The LRX family illustrates the conservation of an archetype of chimeric extensin which appeared in Chlorophyceae

The chimeric structure of the LRX proteins is reminiscent of cell wall structural proteins of *Chlorophyceae*. The volvocalean cell wall is mostly composed of proteins where hydroxyproline-rich-glycoproteins are very abundant. A conserved feature of these HRGPs is their particular conformation consisting of a rigid shaft and a globular domain (Woessner et al. 1994). The algal-cell adhesion molecule (algal-CAM) of *Volvox*, the first plant homologue of an animal cell adhesion molecule, is a chimeric HRGP containing a N-terminal extensin-like domain and two repeats homologous to fasciclin I of *Drosophila*. This protein is required for cell aggregation in the multicellular *Volvox* algae (Huber 1994). Another HRGP (the inversion-specific glycoprotein; ISG) with a cysteine-rich N-terminal domain was also shown to be rapidly and specifically induced during the inversion process of the *Volvox* embryo (Ertl et al. 1992). Electron microscopic analysis of these proteins revealed that they also adopt the rod-knob configuration. Agglutinins are other algal HRGPs implicated in *Chlamydomonas* sexual mating. Athough agglutinin genes have not yet been cloned, their amino acid composition and their structure as visualized with electron microscopy strongly suggest that they are extensin chimeras (Adair et al. 1983; Cooper et al. 1983). The volvocalean HRGPs may thus constitute an archetype of chimeric plant extensins illustrating how the extensin motif has evolved from its initial function as structural component of the vegetative cell wall to a function as anchoring domain for adhesive or signaling proteins (algal-CAM, ISG, sexual agglutinins). With the specialization of the higher plant cell wall, this latter function has perhaps been conserved and adapted to a new structural environment. It is interesting to note that 4 of the 11 *LRX* genes are expressed in flowers and more precisely in pollen, as demonstrated by promoter::GUS experiment for three of them (*LRX3*, *LRX4* and *LRX9*; data not

shown). Such an overrepresentation in a single cell type might suggest redundancy in a crucial function but could also indicate the specialization of some of the pollen *LRX* genes for intercellular signaling during pollination whereas others are required for proper cell expansion, like *LRX1*.

5.7 LRX1 *is specifically expressed in trichoblasts*

pLRX1::GUS analysis indicated that *LRX1* expression is strongly correlated with root hair development either in the collet region or along the differentiation zone. This was strongly supported by the fact that the expression of the endogenous *LRX1* gene was also considerably reduced in seedlings that were inhibited in root hair formation, either as the result of a mutation (i.e. the *rhd6* mutant) or of treatment with the ethylene biosynthesis inhibitor AVG. Ethylene is involved in trichoblast specification in the meristem and the current model assumes that epidermal cells at the R position (placed over anticlinal cell wall of the underlying cortex cells) are more exposed to ethylene which diffuses radially between the cortex cells (Tanimoto et al. 1995). Therefore, inhibition of ethylene biosynthesis prevents specification of trichoblasts which then follow the default developmental pathway of atrichoblasts. Instead, in the *rhd6* mutants trichoblasts are correctly defined, as demonstrated by diverse cytological markers, but fail to initiate root hairs (Masucci and Schiefelbein 1994). The absence of *LRX1* expression in *rhd6* mutants suggests that its expression depends not only on trichoblast specification but more directly on the actual root hair formation. Moreover, in the *rhd1*, *rhd2*, *rhd3* and *rhd4* mutants which are affected in root hair morphogenesis, *LRX1* expression is reduced proportionally to the severity of the root hair defect, indicating that *LRX1* expression is continuously regulated during root hair growth.

GUS activity was also observed in the collumella cells of the root cap. This activity was not abolished with AVG treatment as observed in the root differentiation zone. In contrast, no mycLRX1 protein was detected in the root cap of mycLRX1 plants. The mycLRX1 construct, as compared to the pLRX1::GUS construct, contains the *LRX1* coding sequence and 3' non translated sequence, which might contain regulatory elements. Thus, the root

cap GUS activity is likely to be artifactual. This is supported by the absence of any visible defect in these cells in *lrx1* null mutants.

5.8 LRX1 is specifically localized in the root hairs proper

The polyclonal antibodies raised against the LRR-domain recognized a polypeptide of approximately 150 kDa (p150) in a crude protein extract from wild-type plants. Depletion of the antiserum with the recombinant LRX1 protein abolished the signal, indicating that p150 shares common epitopes with LRX1. Anti-LRX1 antibodies, however, still recognized a polypeptide of the same size in a crude extract of the *lrx1* mutants. These observations lead to the conclusion that p150 does not represent exclusively LRX1 but other immunologically related proteins as well and might in fact correspond to a mixture of LRX1 and other LRX proteins. The strong homology observed between the LRR domain of LRX1 and several LRX protein could easily explain cross-reactions with polyclonal antibodies raised against LRX1. This was indeed demonstrated on western blots of crude protein extract of *E.coli* expressing either recLRX1 or recLRX2.

To overcome the difficulties caused by cross-reactivity of the polyclonal antibodies, a protein tagging approach was used. A polypeptide of approximately 160 kDa was detected by anti-c-myc mAbs in plants transformed with the mycLRX1 construct. Since the c-myc tag would increase the mass of the protein by approximately 10 kDa, the size of the mycLRX1 is in agreement with the size of the protein(s) detected with the anti-LRX1 antiserum, confirming that LRX1 is probably also present in the p150 band. The apparent molecular mass of LRX1 is almost twice the expected value as deduced from its primary sequence. The usually intense glycosylation of extensins might be responsible for this shift in size. Using anti-PEX1 antibodies, Rubinstein et al. (1995b) detected both a 300 kDa protein doublet and a higher molecular mass polypeptide which disappeared upon protein deglycosylation. They concluded that the 300 kDa polypeptide was likely to be the non-glycosylated form of PEX1. If these observations are compared with our data on LRX1, the 160 kDa polypeptide may rather represent the non-glycosylated form of LRX1. The absence of higher molecular mass polypeptides might be due to a stronger

insolubilization of LRX1 in the wall compared to PEX1. In such a case, the low mobility of PEX1 and LRX1 in SDS-PAGE could be due to the rod-like conformation of the extensin determined by its primary structure.

Using plants producing the c-myc tagged LRX1, the protein was localized specifically in the root hair proper throughout all stages of development. The whole-mount immunolabelling of mycLRX1 very likely reflects the exact localization of the endogenous LRX1 protein, since the mycLRX1 construct was able to fully complement the *lrx1* mutation. It clearly suggests that the introduction of the c-myc tag at the N-terminus of the LRRs neither prevents interaction with the ligand nor disturbs the correct subcellular targeting of the protein. The absence of labeling in the epidermal part of trichoblast cells is also consistent with the observation that, in *lrx1* mutants, these cells are normal except for the altered hair development.

5.9 The extensin domain is required for insolubilization of LRX1 *in the cell wall and is important for* LRX1 *function*

As extensins are frequently insolubilized in the cell wall upon developmental cues or various stresses (Showalter 1993), it is possible that the function of the extensin domain of LRX1 is to direct the protein to a particular domain within the cell wall and/or to insolubilize it. Indeed, mycLRX1 was immunodetected in the mature root hairs with whole-mount immunolabeling, whereas no signal was detected in the mature zone of the root with tissue print experiments. This suggests that the protein is insolubilized during root hair development. Such a hypothesis is confirmed by the presence of significant amounts of mycLRX1 in a purified cell wall fraction after extensive protein extraction under harsh conditions. In view of this result, LRX1 is probably covalently bound. HRGP insolubilization can be mediated by oxidative cross-linking between tyrosine residues (Fry 1986; Brady and Fry 1997) and Tyr-x-Tyr motifs have been proposed to be sites of intramolecular cross-links (Kieliszewski and Lamport 1994). Initially, intermolecular cross-links were postulated but no experimental data have supported this hypothesis since then. Therefore, other tyrosine-containing motifs must be involved in the formation of intermolecular networks. In LRX1 nine Tyr-x-Tyr triplets and several other single

tyrosine residues which might contribute to the insolubilization of the protein are found. It is noteworthy to mention that the tyrosine residue at the carboxy-terminus of LRX1 is present in almost every LRX protein and is comprised in a short region which, unlike most of the rest of the extensin domain, is well conserved within the family. The carboxy-terminus of LRX proteins might thus be crucial for the correct interaction of the protein with the cell wall.

The even distribution of the LRX1 protein along the root hair proper might be a possible consequence of its insolubilization in the cell wall. As root hair growth is restricted to the apex, LRX1 might actually be translocated to the cell wall and exert its function only at the tip of elongating root hairs. During ongoing growth, the cell wall at the tip becomes part of the lateral wall, resulting in the observed distribution of LRX1. Since it is possible that only the nonglycosylated LRX1 protein can be detected on protein immunoblots, it is probable that the insolubilization of the mature protein occurs rapidly after secretion to the apoplasm.

The importance of the extensin domain for LRX1 function is underscored by the fact that overexpression of an LRX1 protein deprived of its extensin domain (N/LRX1) phenocopies the *lrx1* null mutation, whereas the overexpression of the full-length LRX1 does not result in an altered root hair development. This suggests that N/LRX1 competes for and blocks the interaction sites used by the endogenous LRX1 protein, preventing wild-type activity. Although 35S-LRX1 is also expressed at a much higher level than the endogenous protein, it does not seem to exert the same effect as 35S-N/LRX1, most likely because it merely replaces the endogenous LRX1. A further conclusion can be drawn from these results: assuming that the extensin domain is responsible for cell wall binding, the interaction between the LRR domain and its ligand is only effective if the former is anchored in the cell wall.

5.10 The possible role of LRX1 in cell morphogenesis and cell expansion

The phenotype observed in the *lrx1* mutants is caused by a complete loss of function mutation, since no full length mRNAs could be detected in any of the mutant lines. Because root hair expansion is restricted to the tip, cell morphology largely depends on the proper regulation of the cellular events

occurring at that location. The *lrx1* root hair phenotype might thus be explained by a defective cell expansion, resulting from a spatially deregulated exocytosis or altered deposition of new cell wall material. In this perspective, LRX1 might contribute to establish or stabilize root hair polarization and tip growth by physically connecting the cell wall and the plasma membrane. In *Fucus* embryos, the cell wall is necessary to fix the polarization axis which determines the rhizoid outgrowth (Kropf et al. 1988), suggesting that interactions between the cell wall and the cytoplasm are involved in cell polarization (Fowler and Quatrano 1997). A similar continuum between the extracellular matrix, the plasma membrane and the cytoskeleton has been shown to exist in animal cells (Howe et al. 1998; Aplin et al. 1999) and was suggested in higher plants by indirect evidence (Wyatt and Carpita 1993; Reuzeau and Pont-Lezica 1995; Howe et al. 1998; Aplin et al. 1999). Two classes of proteins, arabinogalactan proteins (AGPs; Schultz et al. 1998) and wall-associated kinases (WAKs; He et al. 1996), have recently been proposed to serve as bridge between the cell wall and the plasma membrane. The cleavage of the GPI anchor of the AGPs, or the activation of the kinase domain of the WAKs might mediate signal transduction from the wall to the cytoplasm. Although LRX1 does not apparently have enzymatic function and does not contain obvious protein cleavage sites which might lead to the release of a processed peptide, direct physical interactions with plasma membrane integral proteins could still potentially transmit information through the plasma membrane.

Root hair polarization and growth orientation have been shown to depend on both, microtubules (MTs) and the $[Ca^{2+}]$ gradient at the tip. While MTs disturbance results in branching and irregular root hair orientation (Bibikova et al. 1999), manipulation or dissipation of the $[Ca^{2+}]$ gradient reorients or stops root hair growth (Bibikova et al. 1997; Wymer et al. 1997). In support of these findings, the *rhd2* mutant root hairs that fail to elongate are also defective in the formation of the $[Ca^{2+}]$ gradient (Wymer et al. 1997). The similarity between the *lrx1* root hair phenotype and the above mentioned abberations identify MTs and membrane Ca^{2+} channels as possible direct or indirect targets for LRX1 action.

Alternatively, LRX1 function might rather be to locally regulate and organize cell wall expansion at the tip of root hairs, either by recruiting enzymes into critical domains of the cell wall or by directly controlling their activity.

Defective regulation of cell wall assembly might indirectly disturb tip growth and result in the deformations observed such as branching or swelling. Investigations on the ultrastructure of the *rhd4* root hairs which share a very similar phenotype with *lrx1* root hairs has revealed irregular increases in cell wall thickness, a possible consequence of a disturbed wall expansion (Galway et al. 1999). Our preliminary ultrastructural data of the *lrx1* root hair cell wall did not show any obvious modification in the cell wall thickness of the *lrx1* root hairs. Instead, we observed in some cases the presence of inclusions in the fibrillar matrix, as well as low density areas, and locally thinner wall zones which might be the sign of an altered cell wall deposition and organization. These first investigations mostly focused on mutant root hairs which, despite their abnormal shape, were apparently still alive before fixation. It is possible that the most severe defects caused by the *lrx1* mutations were not observed because they led to root hair disruption and collapse. Indeed, time-lapse observations as well as the high percentage of very short root hairs indicated that a large proportion of *lrx1* root hairs merely abort by cell wall rupture. This dramatic event is reminiscent of the effect caused by the *kojak* mutation which was recently identified to affect a cellulose synthase-like enzyme (Favery et al. 2001).

In order to better understand the role of LRX1 in root hair development, the phenotype of double mutants was analyzed. The *lrx1rhd2* phenotype is identical to the *rhd2* mutants showing that *RHD2* is epistatic to *LRX1*. This is consistent with the fact that *rhd2* root hairs do not elongate and therefore do not require LRX1 function. *lrx1rhd1*, *lrx1rhd3* and *lrx1rhd4* in contrast showed additive phenotypes since traits of both homozygous parents were observed. The reduced number of root hairs in the double mutants which is a characteristic of the *lrx1* mutation is probably caused by the high proportion of root hair ruptures. This was particularly visible in *lrx1rhd1* mutants where closer examination revealed many traces of collapsed, swollen root hairs. The few root hairs which elongate in the double mutants showed mostly the phenotype of the *rhd* parent. In the case of *lrx1rhd4* mutants, the addition of two very similar phenotypes lets the double mutant appear an enhanced *lrx1* or *rhd4* single mutant. These observations suggest that LRX1, RHD1, RHD3, and RHD4 act via independent mechanisms and confirm that the major effect of the *lrx1*

mutation is the weakening of the root hair cell wall. As the *lrx1* mutation does not provoke root hair rupture in every case, the effects of the *rhd* mutation is essentially visible on the few root hairs which escape abortion and elongate further.

5.11 Future research directions and outlook

To our knowledge, LRX1 is the first extensin-like protein which was analyzed at the functional level, and the results presented above provide the first direct evidence of a role for extensin-like proteins in cell expansion. Therefore, the *lrx1* mutant provides a good system to easily assess, by complementation, the specific contribution of particular sub-domains of the extensin domain to protein function. Comparison of the different members of the *LRX* family has revealed the presence of conserved and variable motifs whose biological relevance can be tested by domain swapping and deletion experiments. Similar experiments could also be used to investigate the ligand specificity of the LRR domains. The data obtained from such experiments may help to understand better how discrimination in protein-protein interaction is achieved in LRRs. The functional characterization of the other *LRX* genes should also provide considerable insight into the role of these chimeric proteins in cell morphogenesis and answer two main questions: Are they specifically required for directional cell expansion? Are they involved in other cell wall related processes such as cell signaling? Considering the risk of functional redundancy, multiple mutants or an RNA interference approach will possibly be necessary. Since no phenotype was observed by either knocking out the *LRX2* gene or overexpressing its LRR domain, an approach which was successful with *LRX1*, the function of *LRX2* remains puzzling and deserves some attention. The elucidation of the molecular function of *LRX1* will greatly benefit from protein interaction studies of the LRR domain of *LRX1*. A yeast two hybrid approach or immunoprecipitation with tagged recombinant LRX1 unable to crosslink to the cell wall, might lead to the identification of the interacting partner of LRX1. The localization of the protein at the ultrastructural level should help us to discriminate between the hypotheses formulated above. If LRX1 indeed controls targeted exocytosis and polar growth by interacting with the plasma

membrane, its sublocalization in the cell wall should be consistent with this function. Further characterization of the *lrx1* mutant at the ultrastructural level using antibodies against different cell wall components, might also identify more precisely the role of LRX1 during root hair growth.

5.12 Conclusion

In this work we identified a new family of LRR-containing proteins with an extensin domain. This family is present both in monocots and dicots and is therefore likely to play an important role in the plant kingdom. The functional characterization of *LRX1,* the first gene of this family identified in *Arabidopsis*, has demonstrated that it is important for root hair expansion and morphogenesis. LRX1 represents one of the very few genes involved in root hair morphogenesis which have been cloned so far and is the first cell wall structural-like protein to be investigated at the functional level. LRR-extensin might constitute a new major determinant of cell shape in plants.

6. Material and methods

6.1 Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia was used for all experiments. The *rhd1, rhd2, rhd3* and *rhd4* mutants were obtained from the *Arabidopsis* Biological Resource Center. *rhd6* was a gift of J. Schiefelbein (University of Michigan). The *lrx1* and *lrx2* mutants were isolated from an *En-1* mutagenized *Arabidopsis* population (Wisman et al. 1998) by PCR screening as reported in Baumann et al. (1998) using the *LRX1* and *LRX2* gene specific primers lrx1MUT1f, lrx1MUT1r, lrx1MUT2f, lrx1MUT2r, lrx2MUT1f, lrx2MUT1r, lrx2MUT2f, lrx2MUT2r and probes derived either from the *LRX1* cDNA fragment 332-1148 spanning the LRR domain (referred to as *LRX1* probe) or from the *LRX2* sequence fragment 255-1322 (referred to as *LRX2* probe). The precise positions of the *En-1* insertions were obtained by cloning and sequencing the PCR products spanning the right and left border of the *En-1* element. Mutant plants were backcrossed several times (four times in the case of *lrx1* mutants) with wild-type plants to remove additional insertions.

Plants were grown in soil, under continuous light at 24°C in growth chambers. Alternatively, seeds were surface-sterilized, stratified in darkness for 2-3 d at 4°C and grown vertically at 24°C, under continuous illumination, along the surface of half-strength MS medium, supplemented with 2% sucrose and 0.6% Phytagel (Sigma). Transgenic seedlings were selected on half-strength MS plates with 0.8% Phytoagar (Gibco BRL) supplemented with 50 µg/ml kanamycine. All experiments using transgenic lines were performed in the T_3 or T_4 generation unless specified.

6.2 DNA primers

The following DNA primers were used for plasmid constructs, probe amplification, and screening of the *En-1* mutagenized population. The numbers in parentheses refer to the position of the 5'-end of the *LRX1* and *LRX2* sequences (see Appendix)

***LRX1* primers:**

lrx1f: 5'-AGTCGTTGCTGGCATTGACC-3' (332),

lrx1r:5'-ATCCACAGGGCGAGCAAGC-3' (1148),

p1GUSf: 5'-TATGAACTTACCATTCCAAGC-3' (-1562),

p1GUSr: 5'-GAAGATCTAGAGGGAACGAAGAGGAGGGA-3' (71),

lrx1MUT1f: 5'-GACCACGACGATGAAAGCGACTTTTGG-3' (114),

lrx1MUT2f: 5'-AGATCGGAAACCTCAAGAAAGTGACG-3' (817),

lrx1MUT1r: 5'-AGCTCGAACGCTAGGTGACATCTTGG-3' (1700),

lrx1MUT2r: 5'-CATATGAGACGCTTGGCATCGGTGG-3' (2235),

MYCf: 5'-CCCCCCCGAGGTCGACGGTATC-3',

MYCr: 5'-ATCCCTCGGGATCGATTTCGAACC-3',

35SLRX1f: 5'-TAAAATGAATTCTCTTGACCCATAAGC-3' (8),

35SLRX1r: 5'-AAAGTTCTAGATTGTGAGTAGTCTCG-3' (2337),

35SN/LRX1r: 5'-CAAAGATCTAGACTGTTTATCCGATC-3' (1130),

recLRX1f:5'-CTGCATGCTACCCGAAAACCCGAGTCG-3' (310),

recLRX1r: 5'-AGCTGCAGCCGGTGATGCAGTTCATGG-3' (1097).

***LRX2* primers:**

lrx2MUT1f: 5'-GTTGTTTCCTTCTACTTCTTTACGGTCTC-3' (135)

lrx2MUT1r: 5'-GGAGTTATACCAAGCAGCATTTGTCAG-3' (1590)

lrx2MUT2f: 5'-ATGCCCTAACGGAAGGTGACATTTCG-3' (1047)

lrx2MUT2r: 5'-GATAGGCGGAAGAGGTGTGTCTTCG-3' (2441)

p2GUSf: 5'-AAAAGCTTTAGTTGGAGGTTAATTTACGC-3' (-1370)

p2GUSr 5'-AATCTAGAAGAGACGTAAAGAAGTAGAAG-3' (172)

35SLRX1f: 5'-TCAATTGAATTCAACTCTTGAAACTAAG-3' (97)

35SN/LRX1r: 5'-CTTACTTCTAGAAACAGAGCGTGAG-3' (1255)

35SLRX1r: 5'-AACCATTCTAGACAAATAGGTTGATGC-3' (2522)

recLRX2f:5'-CTGCATGCCCGAAAACTCGAGTTGGTTGC-3' (418),

recLRX2r: 5'-CTCTGCAGAACAGAGCGTGAGGCTGG-3' (1255)

6.3 LRX1 and LRX2 genomic clone isolation and RACE-PCR

An EMBL3 *Arabidopsis thaliana* genomic library (Clontech) was screened with the ^{32}P-labeled *LRX1* probe. A *Sph*I-*Nco*I genomic fragment containing the *LRX1* gene with 1.8 kb of 5'- and 0.83 kb of 3'-untranslated sequence was

isolated, cloned and sequenced to confirm its identity (referred to as pλLRX1). The actual transcription start site of the *LRX1* gene, the polyadenylation site and the cDNA sequence were determined by 5'- and 3'- RACE-PCR using the GeneRacer Kit (Invitrogen) and the gene-specific primers MUT1r and MUT2f. The resulting PCR products were cloned and sequenced (8 independent clones for each PCR product). The *LRX1* cDNA sequence has been deposited with GenBank under the following accession number: AY026364.

A lambda ZAPII library of *Arabidopsis thaliana* genomic DNA (Stratagene) was screened with the ^{32}P-labeled LRX1 probe. An *Eco*RI genomic fragment containing the *LRX2* gene with 1.55 kb of 5'- and 2.63 kb of 3'-untranslated sequence was isolated, cloned and sequenced to confirm its identity (referred to as pλLRX2).

6.4 Constructs and plant transformation

For the *LRX1* and *LRX2* promoter*::GUS* fusion constructs (*pLRX1::GUS, pLRX2::GUS*), 1.6 and 1.5 kb of the promoter region were amplified by PCR from pλLRX1 and pλLRX2 with the primer pairs p1GUSf/p1GUSr and p2GUSf/p2GUSr, respectively, digested with *Xba*I and *Hin*dIII, and cloned into pGPTV-KAN (Becker et al. 1992). For expression of the c-myc-tagged LRX1 protein (*mycLRX1* construct), a 6-fold duplicated copy of the human c-myc epitope (EQKLISEEDL) sequence was amplified by PCR from the vector CD3-128 (*Arabidopsis* Biological Resource Center) using the primers MYCf and MYCr, digested with *Ava*I and ligated into the single *Ava*I restriction site of pλLRX1 (position 329 in the *LRX1* cDNA). A clone with the c-myc tag in the sense orientation was selected, linearized with *Not*I and subcloned into pART27 (Gleave 1992). For constitutive expression of the full-length (35S-LRX1) and truncated (35S-N/LRX1) LRX1 proteins, the *LRX1* coding sequences were amplified by PCR from pλLRX1 with the primer combinations 35SLRX1f / 35SLRX1r and 35SLRX1f / 35SN/LRX1r, digested with *Eco*RI and *Xba*I and cloned into pART7. For constitutive expression of the full-length (35S-LRX2) and truncated (35S-N/LRX2) LRX2 proteins, the *LRX2* coding sequences were amplified by PCR from pλLRX2 with the primer combinations 35SLRX2f / 35SLRX2r and 35SLRX2f / 35SN/LRX2r, digested with *Eco*RI and *Xba*I and

cloned into pART7. The expression cassette of pART7 was then subcloned into the binary vector pART27 (Gleave 1992).

The T-DNA constructs were transformed into *Agrobacterium tumefaciens* GV3101 and plant transformation was performed following the floral dip method described by Clough and Bent (1998). Transgenic plants were selected on MS agar plates containing kanamycine and the number of segregating T-DNA loci was assessed by segregation of the kanamycine resistance in the next generation. Resistant plants were transferred to soil and selected to obtain T_3 homozygous seeds.

6.5 GUS histochemical analysis

Histochemical staining for GUS activity was performed by incubation in 50 mg/ml 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc), in 50 mM Na-phosphate buffer, pH 6.8, 10 mM EDTA, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, 0.1% [v/v] Triton X-100, at 37°C for 16 hr. For AVG treatments, plants were grown on the surface of normal MS medium for three days, transferred onto MS plates supplemented with 20 µM of L-α-(2-amino-ethoxyvinyl)glycine (Sigma) and grown for 5 additional days before histochemical staining or Northern analysis. As control, plants were transferred onto MS plates without AVG and subsequently grown for 5 days.

6.6 Protein immunoblotting

Plant material was ground in liquid nitrogen and proteins were extracted on ice with 0.5 M $CaCl_2$ in 100 mM HEPES at pH 7.5, 10 mM DTT, 0.5 mM EDTA and proteinase inhibitors (Complete mini, Boehringer-Mannheim). Proteins were precipitated with deoxycholic acid/ trichloroacetic acid and resuspended in Laemmli buffer. Alternatively, proteins were directly extracted in Laemmli buffer at 100°C for 5 min (35S-N/LRX1and 35S-N/LRX2 extracts). Proteins were resolved by 6% to 8% SDS-PAGE and blotted onto PVDF Immobilon-P membrane (Millipore), using the Mini Trans-Blot transfer cell (BioRad). Transfer was performed at 10 mA, for 16 hr at 4°C, in 0.1% [w/v] SDS, 25 mM Tris, 192 mM glycine and 15% [v/v] methanol. Alternatively (35S-N/LRX1 extract), transfer was done in the Trans-Blot SD semi-dry transfer cell

(BioRad), in 0.0375% [w/v] SDS, 48 mM Tris-HCl, 39 mM glycine, 20% [v/v] methanol at 0.8 mA/cm^2 of membrane for 30 minutes. Membranes were blocked for 1 hr in 20 mM Tris, pH 7.6, 137 mM NaCl, 0.1 % [v/v] Tween-20 (TBS-T) with 5% [w/v] non-fat milk. Primary antiserum dilutions were as follows: rabbit anti-LRX1or anti-LRX2 IgG, 1:10,000; mouse anti-c-myc 9E10.2, 1:5,000 (Abcam). Secondary antiserum dilutions were as follows: goat anti-mouse horseradish peroxidase-conjugated IgG, 1:5,000 (BioRad); goat anti-rabbit horseradish peroxidase-conjugated IgG, 1:20,000 (Sigma). All antibodies were diluted in TBS-T, incubations were done at room temperature for 1 hr and were followed by four washes of 15 min in TBS-T. Chemiluminescence detection was performed with ECL+ reagents according to the recommendations of the manufacturer (Amersham).

6.7 Tissue print, whole-mount and cell wall immunolocalization

Tissue-print experiments were performed as described by Cassab (1992). Immunodetection was performed with mouse anti-c-myc 9E10.2, 1:500 (Abcam) and rabbit anti-mouse alkaline phosphatase-conjugated antibodies, 1:1,000 (Boehringer Mannheim) followed by detection using the alkaline phosphatase conjugate substrate of BioRad.

Whole-mount immunolocalizations were done with 3-day-old seedlings, which were fixed for 30 min at room temperature in 4% [w/v] paraformaldehyde, 50 mM HEPES, pH 7.0, rinsed 4 times in HEPES buffer, and 6 hr in 2% [w/v] BSA, 4x SSC. Incubations with antisera diluted in 4x SSC, 2% [w/v] BSA were performed at 4°C for 16 hr (primary antibodies: mouse anti-c-myc 9E10.2, 1:1,000 (Abcam); secondary antibodies: goat anti-mouse 1nm gold-conjugate IgG, 1:1,000 (British BioCell International)) and followed by four washes of 1 hr each in 4x SSC, 0.05% [v/v] Tween-20. Finally, samples were rinsed four times in ultra-pure water for 15 min each, and the signal was amplified with silver enhancement reagents (British BioCell International). Observations were made with a Leitz Laborlux microscope equipped with epipolarized illumination.

Cell wall preparations were obtained by grinding fresh root material from 14-day-old seedlings in liquid nitrogen followed by extraction in phenol:acetic acid according to Fry (1988). The purified cell walls were resuspended in 80%

acetone and laid down on Biobond-coated microscope slides (British BioCell International). Immunodetection was then performed on the slides as follows: material was blocked for 2 hr in TBS-T, 10% [v/v] normal goat serum (British BioCell International), 5% [w/v] BSA. Incubations with antisera diluted in TBS-T, 1% [v/v] normal goat serum, 1% [w/v] BSA, were done for 2 hr at room temperature (primary antibodies: mouse anti-c-myc 9E10.2, 1:200 (Abcam); secondary antibodies: goat anti-mouse 1nm gold-conjugate IgG , 1:250 (British BioCell International)) and followed by three washes of 10 min each in TBS-T. Three additional washes of 3 min in pure water were performed before amplification of the signal with silver enhancement reagents (British BioCell International).

6.8 Microscopical observations

Light microscopical observations were done with a Leica stereomicroscope LZ M12$_5$. For scanning electron microscopy, seedlings grown on the surface of MS medium were transferred onto humid nitrocellulose membranes on metal stabs and rapidly frozen in liquid nitrogen. Samples were partly freeze-dried in high vacuum (<$2x10^{-4}$ Pa) at –90°C for 30 min and sputter coated with platinum in a preparation chamber SCU 020 (BAL-TEC) before observation at –120°C in a SEM 515 scanning electron microscope (Philips) .

6.9 Production of polyclonal antibodies

The sequences encoding the LRR domain of LRX1and LRX2 were amplified by PCR using the primer combination recLRX1f/recLRX1r and recLRX2f/recLRX2r, respectively. Purified PCR products were digested with *Sph*I and *Pst*I, cloned into the expression vector pQE-30 (Qiagen) and transformed into *E.coli* BL21. Single colonies were selected and grown to an OD_{600} of 0.6 before induction with 1 mM IPTG for 4 hr at 37°C. Cells were collected by centrifugation, lysed in 5 volumes of 8 M urea, 0.1 M Na_2HPO_4, 0.01 M Tris, pH 8, for 2 hr at room temperature and lysates were cleared by centrifugation (30 min, 10,000 *g*). The recombinant proteins recLRR1 and recLRR2, fused to a 6xHis tag, were recovered and purified by affinity chromatography on a Ni-NTA agarose bead column (Qiagen). Two rabbits were

immunized with each purified recLRR1 and recLRR2 proteins, IgG were purified by protein A affinity chromatography and used for immunoblotting.

6.10 DNA and protein sequence analysis

Sequence alignments, translation, dendrogram representation, and pairwise identity calculation were performed with the GCG Sequence Analysis Software package Version 10 (Devereux et al. 1984) using the PILEUP, MAP, and DISTANCE programs. Putative open reading frames were determined by hand using the Arabidopsis genome annotations at TAIR as starting material. Prediction of the signal peptide was performed with the SignalP V1.1 program (Nielsen et al. 1997).

6.11 Anatomic and ultrastructure analysis

Three-day old seedlings grown at the surface of MS medium were rapidly frozen in liquid propane at -120°C, and freeze-substituted in 1% OsO_4 in methanol for 3 d at -80°C before gradually rewarming over a 12 h period. At 0°C the samples were given three changes of pure methanol to remove unreacted OsO_4. The roots were then infiltrated with a graded series of Spurr resin and polymerized in thin layers at 60°C for 72 h. After orientation the blocks were reembedded in Spurr resin or glued on supports for sectioning. For light microscopy, semi-thin sections were cut on a glass knife at 1 µm and stained with toluidine blue before mounting in Eukitt. For transmission electron microscopy, thin sections of approximately 70 nm were obtained with an Ultracut E microtome (Leica) and stained with uranyl acetate (2% in 50% acetone) and alkaline lead citrate for 30 min each. Micrographs were taken with a Philips CM 100 BIOTWIN electron microscope at 80 kV using a 30 μm objective diaphragm.

6.12 Isolation of double mutants

lrx1 mutant plants were crossed with *rhd1*, *rhd2*, *rhd3*, and *rhd4* mutants and 10 plants of the F_2 progeny which displayed the phenotype of the *rhd* parent or a more severe phenotype than the *lrx1* parent were selected, self-fertilized to produce a F_3 generation and crossed with the *rhd* parent. The

genotype at the *LRX1* locus was determined by Southern blot RFLP analysis of DNA isolated from a leaf of each plant of the F_2 progeny and the genotype at the *RHD* locus was assessed by examination of the phenotype in progenies resulting from the backcross with the *rhd* parent.

6.13 *Measurement of root hair parameters*

Measurements of root hair parameters were made on 3-day-old seedlings, grown at the surface of MS mediumm, under a binocular equipped with a micrometer ocular. Hair density was measured on 1 mm of mature root by counting only hairs growing in the air. The hair length was measured on five randomly selected mature wild-type root hairs or mutant root hairs, respectively, growing parallel to the medium surface. The percentage of hairs longer than 40 μm, branched root hairs and swollen hairs was determined on the same root portion used to measure the hair density.

7. Bibliography

Adair, W.S. and K.E. Apt. 1990. Cell wall regeneration in *Chlamydomonas*: accumulation of mRNAs encoding cell wall hydroxyproline-rich glycoproteins. *Proc. Natl. Acad. Sci. USA.* **87**: 7355-7359.

Adair, W.S., C. Hwang, and U.W. Goodenough. 1983. Identification and visualization of the sexual agglutinin from the mating-type plus flagellar membrane of *Chlamydomonas. Cell* **33**: 183-193.

Akashi, T. and H. Shibaoka. 1991. Involvement of transmembrane proteins in the association of cortical microtubules with the plasma membrane in tobacco By-2 cells. *J. Cell Sci.* **98**: 169-174.

Aplin, A.E., A.K. Howe, and R.L. Juliano. 1999. Cell adhesion molecules, signal transduction and cell growth. *Curr. Opin. Cell Biol.* **11**: 737.

Arioli, T., L.C. Peng, A.S. Betzner, J. Burn, W. Wittke, W. Herth, C. Camilleri, H. Hofte, J. Plazinski, R. Birch, A. Cork, J. Glover, J. Redmond, and R.E. Williamson. 1998. Molecular analysis of cellulose biosynthesis in *Arabidopsis. Science* **279**: 717-720.

Balestrini, R., M. Jose-Estanyol, P. Puigdomenech, and P. Bonfante. 1997. Hydroxyproline-rich glycoprotein mRNA accumulation in maize root cells colonized by an arbuscular mycorrhizal fungus as revealed by in situ hybridization. *Protoplasma* **198**: 36-42.

Baluska, F., J. Salaj, J. Mathur, M. Braun, F. Jasper, J. Samaj, N.H. Chua, P.W. Barlow, and D. Volkmann. 2000. Root hair formation: F-actin-dependent tip growth is initiated by local assembly of profilin-supported F-actin meshworks accumulated within expansin-enriched bulges. *Dev. Biol.* **227**: 618-632.

Battey, N.H., N.C. James, A.J. Greenland, and C. Brownlee. 1999. Exocytosis and endocytosis. *Plant Cell* **11**: 643-659.

Baumann, E., J. Lewald, H. Saedler, B. Schulz, and E. Wisman. 1998. Successful PCR-based reverse genetic screens using an *En-1*-mutagenised *Arabidopsis thaliana* population generated via single-seed descent. *Theor. Appl. Genet.* **97**: 729-734.

Becker, D., E. Kemper, J. Schell, and R. Masterson. 1992. New plant binary vectors with selectable markers located proximal to the left T-DNA border. *Plant Mol. Biol.* **20**: 1195-1197.

Berger, F., J. Haseloff, J. Schiefelbein, and L. Dolan. 1998. Positional information in root epidermis is defined during embryogenesis and acts in domains with strict boundaries. *Curr. Biol.* **8**: 421-430.

Bernhardt, C. and M.L. Tierney. 2000. Expression of *AtPRP3,* a proline-rich structural cell wall protein from *Arabidopsis*, is regulated by cell-type-specific developmental pathways involved in root hair formation. *Plant Physiol.* **122**: 705-714.

Bibikova, T.N., E.B. Blancaflor, and S. Gilroy. 1999. Microtubules regulate tip growth and orientation in root hairs of *Arabidopsis thaliana. Plant J.* **17**: 657-665.

Bibikova, T.N., A. Zhigilei, and S. Gilroy. 1997. Root hair growth in *Arabidopsis thaliana* is directed by calcium and an endogenous polarity. *Planta* **203**: 495-505.

Bonilla, I., C. Mergold-Villasenor, M.E. Campos, N. Sanchez, H. Perez, L. Lopez, L. Castrejon, F. Sanchez, and G.I. Cassab. 1997. The aberrant cell walls of boron-deficient bean root nodules have no covalently bound hydroxyproline/proline-rich proteins. *Plant Physiol.* **115**: 1329-1340.

Bradley, D.J., P. Kjellbom, and C.J. Lamb. 1992. Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defense response. *Cell* **70**: 21-30.

Brady, J.D. and S.C. Fry. 1997. Formation of di-isodityrosine and loss of isodityrosine in the cell walls of tomato cell suspension cultures treated with fungal elicitors or H_2O_2. *Plant Physiol.* **115**: 87-92.

Braun, M., F. Baluska, M. von Witsch, and D. Menzel. 1999. Redistribution of actin, profilin and phosphatidylinositol-4,5- bisphosphate in growing and maturing root hairs. *Planta* **209**: 435-443.

Brisson, L.F., R. Tenhaken, and C. Lamb. 1994. Function of oxidative cross-linking of cell wall structural proteins in plant disease resistance. *Plant Cell* **6**: 1703-1712.

Brownlee, C. and A.L. Pulsford. 1988. Visualization of the cytoplasmic Ca^{2+} gradient in *Fucus serratus* rhizoids: correlation with cell ultrastructure and polarity. *J. Cell Sci.* **91**: 249-256.

Bucher, M., B. Schroeer, L. Willmitzer, and J.W. Riesmeier. 1997. Two genes encoding extensin-like proteins are predominantly expressed in tomato root hair cells. *Plant Mol. Biol.* **35**: 497-508.

Carpita, N.C. and D.M. Gibeaut. 1993. Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *Plant J.* **3**: 1-30.

Cassab, G.I. 1998. Plant cell wall proteins. *Annu. Rev. Plant Physiol. Plant Mol Biol.* **49**: 281-309.

Cassab, G.I. 1992. localization of cell wall protein. In *Tissue Printing* (ed. P.D. Reid and R.F. Pont-Lezica), pp. 23-40. Academic Press, San Diego.

Chen, C.G., E.C. Cornish, and A.E. Clarke. 1992. Specific expression of an extensin-like gene in the style of *Nicotiana alata*. *Plant Cell* **4**: 1053-62.

Chen, C.G., Z.Y. Pu, R.L. Moritz, R.J. Simpson, A. Bacic, A.E. Clarke, and S.L. Mau. 1994. Molecular cloning of a gene encoding an arabinogalactan protein from pear (*Pyrus communis*) cell suspension culture. *Proc. Natl. Acad. Sci. USA.* **91**: 10305-10309.

Chen, J. and J. Varner. 1985. Isolation and characterization of cDNA clones for carrot extensin and a proline-rich 33-kDa protein. *Proc. Natl. Acad. Sci. USA.* **82**: 4399-4403.

Clark, S.E. 2001. Meristems: Start your signaling. *Curr. Opin. Plant Biol.* **4**: 28-33.

Clark, S.E., R.W. Williams, and E.M. Meyerowitz. 1997. The *CLAVATA1* gene encodes a putative receptor kinase that controls shoot and floral meristem size in *Arabidopsis*. *Cell* **89**: 575-585.

Clough, S.J., and A.F. Bent. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.***16**: 735-743.

Condit, C.M. 1993. Developmental expression and localization of petunia glycine- rich protein-1. *Plant Cell* **5**: 277-288.

Cooper, J.B., W.S. Adair, R.P. Mecham, J.E. Heuser, and U.W. Goodenough. 1983. *Chlamydomonas* agglutinin is a hydroxyproline-rich glycoprotein. *Proc. Natl. Acad. Sci. USA.* **80**: 5898-5901.

Cosgrove, D.J. 2000. Loosening of plant cell walls by expansins. *Nature* **407**: 321-326.

De Lorenzo, G. and F. Cervone. 1997. Polygalacturonase-inhibiting proteins (PGIPs): their role in specificity and defense against pathogenic fungi. In *Plant-Microbe Interactions* (ed. G. Stacey and N.T. Keen). Chapman & Hall, New-York.

Desiderio, A., B. Aracri, F. Leckie, B. Mattei, G. Salvi, H. Tigelaar, J.S.C. VanRoekel, D.C. Baulcombe, L.S. Melchers, G. De Lorenzo, and F. Cervone. 1997. Polygalacturonase-inhibiting proteins (PGIPs) with different specificities are expressed in *Phaseolus vulgaris*. *Mol. Plant-Microbe Interact.* **10**: 852-860.

Devereux, J., P. Haeberli, and O. Smithies. 1984. A comprehensive set of sequence-analysis programs for the vax. *Nucleic Acids Res.* **12**: 387-395.

Devoto, A., A.J. Clark, L. Nuss, F. Cervone, and G. De Lorenzo. 1997. Developmental and pathogen-induced accumulation of transcripts of polygalacturonase-inhibiting protein in *Phaseolus vulgaris* L. *Planta* **202**: 284-292.

Ding, L. and J.K. Zhu. 1997. A role for arabinogalactan-proteins in root epidermal cell expansion. *Planta* **203**: 289-294.

Dolan, L., C. Duckett, C. Grierson, P. Linstead, K. Schneider, E. Lawson, C. Dean, S. Poethig, and K. Roberts. 1994. Clonal relationships and cell patterning in the root epidermis of *Arabidopsis*. *Development* **120**: 2465-2474.

Du, H., A.E. Clarke, and A. Bacic. 1996. Arabinogalactan-proteins: a class of extracellular matrix proteoglycans involved in plant growth develoment. *Trends Cell Biol.* **6**: 411-414.

Edwards, M.E., C.A. Dickson, S. Chengappa, C. Sidebottom, M.J. Gidley, and J.S.G. Reid. 1999. Molecular characterisation of a membrane-bound galactosyltransferase of plant cell wall matrix polysaccharide biosynthesis. *Plant J.* **19**: 691-697.

Ellis, J., P. Dodds, and T. Pryor. 2000. Structure, function and evolution of plant disease resistance genes. *Curr. Opin. Plant Biol.* **3**: 278-284.

Ellis, J. and D. Jones. 1998. Structure and function of proteins controlling strain-specific pathogen resistance in plants. *Curr. Opin. Plant Biol.* **1**: 288-293.

Epstein, L. and D.T.A. Lamport. 1984. An intramolecular linkage involving isodityrosine in extensin. *Phytochemistry* **23**: 1241-1246.

Ertl, H., A. Hallmann, S. Wenzl, and M. Sumper. 1992. A novel extensin that may organize extracellular matrix biogenesis in *Volvox carteri*. *EMBO J.* **11**: 2055-2062.

Fagard, M., T. Desnos, T. Desprez, F. Goubet, G. Refregier, G. Mouille, M. McCann, C. Rayon, S. Vernhettes, and H. Hofte. 2000. *PROCUSTE1* encodes a cellulose synthase required for normal cell elongation specifically in roots and dark-grown hypocotyls of *Arabidopsis*. *Plant Cell* **12**: 2409-2423.

Favery, B., E. Ryan, J. Foreman, P. Linstead, K. Boudonck, M. Steer, P. Shaw, and L. Dolan. 2001. *KOJAK* encodes a cellulose synthase-like protein required for root hair cell morphogenesis in *Arabidopsis*. *Genes Dev.* **15**: 79-89.

Felle, H.H. and P.K. Hepler. 1997. The cytosolic Ca^{2+} concentration gradient of *Sinapis alba* root hairs as revealed by Ca^{2+}-selective microelectrode tests and fura-dextran ratio imaging. *Plant Physiol.* **114**: 39-45.

Fletcher, L.C., U. Brand, M.P. Running, R. Simon, and E.M. Meyerowitz. 1999. Signaling of cell fate decisions by CLAVATA3 in *Arabidopsis* shoot meristems. *Science* **283**: 1911-1914.

Fowler, J.E. and R.S. Quatrano. 1997. Plant cell morphogenesis: plasma membrane interactions with the cytoskeleton and cell wall. *Annu. Rev. Cell Dev. Biol.* **13**: 697-743.

Fowler, T.J., C. Bernhardt, and M.L. Tierney. 1999. Characterization and expression of four proline-rich cell wall protein genes in *Arabidopsis* encoding two distinct subsets of multiple domain proteins. *Plant Physiol.* **121**: 1081-1091.

Freshour, G., R.P. Clay, M.S. Fuller, P. Albersheim, A.G. Darvill, and M.G. Hahn. 1996. Developmental and tissue-specific structural alterations of the cell wall polysaccharides of *Arabidopsis thaliana* roots. *Plant Physiol.* **110**: 1413-1429.

Fry, S.C. 1982. Isodityrosine, a new cross-linking amino acid from plant cell wall glycoprotein. *Biochem. J.* **204**: 449-455.

Fry, S.C. 1986. Cross-linking of matrix polymers in the growing cell walls of angiosperms. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **37**: 165-86.

Fry, S.C. 1988. The growing plant cell wall: chemical and metabolic analysis. Longman Scientific & Technical, Harlow, UK.

Galway, M.E., J.W. Heckman, and J.W. Schiefelbein. 1997. Growth and ultrastructure of *Arabidopsis* root hairs: The *rhd3* mutation alters vacuole enlargement and tip growth. *Planta* **201**: 209-218.

Galway, M.E., D.C. Lane, and J.W. Schiefelbein. 1999. Defective control of growth rate and cell diameter in tip- growing root hairs of the *rhd4* mutant of *Arabidopsis thaliana*. *Can. J. Bot.* **77**: 494-507.

Galway, M.E., J.D. Masucci, A.M. Lloyd, V. Walbot, R.W. Davis, and J.W. Schiefelbein. 1994. The *TTG* gene is required to specify epidermal-cell fate and cell patterning in the *Arabidopsis* root. *Dev. Biol.* **166**: 740-754.

Gandolfo, M.A., K.C. Nixon, and W.L. Crepet. 1998. A new fossil flower from the Turonian of New Jersey: *Dressiantha bicarpellata* gen. et sp. nov. (Capparales). *Am. J. Bot.* **85**: 964-974.

Garcia-Gomez, B.I., F. Campos, M. Hernandez, and A.A. Covarrubias. 2000. Two bean cell wall proteins more abundant during water deficit are high in proline and interact with a plasma membrane protein. *Plant J.* **22**: 277.

Garcia-Muniz, N., J.A. Martinez-Izquierdo, and P. Puigdomenech. 1998. Induction of mRNA accumulation corresponding to a gene encoding a cell wall hydroxyproline-rich glycoprotein by fungal elicitors. *Plant Mol. Biol.* **38**: 623-632.

Garrill, A., S.I. Jackson, R.R. Lew, and I.B. Heath. 1993. Ion channel activity and tip growth: tip localized stretch activated channels generate an

essential Ca^{2+} Gradient in the oomycete *Saprolegnia ferax*. *Eur. J. Cell Biol.* **60**: 358.

Gertel, E.T. and P.B. Green. 1977. Cell growth pattern and wall microfibrillar arrangement. *Plant Physiol.* **60**: 247-254.

Gleave, A.P. 1992. a versatile binary vector system with a T-DNA organisational structure conducive to efficient integration of cloned DNA into the plant genome. *Plant Mol. Biol.* **20**: 1203-1207

Goldman, M.H., M. Pezzotti, J. Seurinck, and C. Mariani. 1992. Developmental expression of tobacco pistil-specific genes encoding novel extensin-like proteins. *Plant Cell* **4**: 1041-1051.

Goodwin, W., J.A. Pallas, and G.I. Jenkins. 1996. Transcripts of a gene encoding a putative cell wall-plasma membrane linker protein are specifically cold-induced in *Brassica napus*. *Plant Mol. Biol.* **31**: 771-781.

Grierson, C.S., K. Roberts, K.A. Feldmann, and L. Dolan. 1997. The *COW1* locus of *Arabidopsis* acts after *RHD2*, and in parallel with *RHD3* and *TIP1*, to determine the shape, rate of elongation, and number of root hairs produced from each site of hair formation. *Plant Physiol.* **115**: 981-990.

Hadfield, K.A. and A.B. Bennett. 1998. Polygalacturonases: many genes in search of a function. *Plant Physiol.* **117**: 337-343.

Hammond-Kosack, K.E. and J.D.G. Jones. 1997. Plant disease resistance genes. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48**: 575-607.

He, Z.H., M. Fujiki, and B.D. Kohorn. 1996. A cell wall-associated, receptor-like protein kinase. *J. Biol. Chem.* **271**: 19789-19793.

He, Z.H., Z.Y. Wang, J.M. Li, Q. Zhu, C. Lamb, P. Ronald, and J. Chory. 2000. Perception of brassinosteroids by the extracellular domain of the receptor kinase BRI1. *Science* **288**: 2360-2363.

Howe, A., A.E. Aplin, S.K. Alahari, and R.L. Juliano. 1998. Integrin signaling and cell growth control. *Curr. Opin. Cell Biol.* **10**: 220-231.

Huber, O., M. Sumper. 1994. Algal-CAMs-Isoforms of a cell-adhesion molecule in embryos of the alga *Volvox* with homology to *Drosophila* fasciclin-I. *EMBO J.* **13**: 4212.

Iozzo, R.V. 1999. The biology of the small leucine-rich proteoglycans: functional network of interactive proteins. *J. Biol. Chem.* **274**: 18843-18846.

Jinn, T.L., J.M. Stone, and J.C. Walker. 2000. HAESA, an *Arabidopsis* leucine-rich repeat receptor kinase, controls floral organ abscission. *Genes Dev.* **14**: 108-117.

Jones, D.A., C.M. Thomas, K.E. Hammond-kosack, P.J. Balintkurti, and J.D.G. Jones. 1994. Isolation of the tomato *Cf-9* gene for resistance to *Cladosporium fulvum* by transposon tagging. *Science* **266**: 789-793.

Keller, B. 1993. Structural cell wall proteins. *Plant Physiol.* **101**: 1127-1130.

Keller, B. and C.J. Lamb. 1989. Specific expression of a novel cell wall hydroxyproline-rich glycoprotein gene in lateral root initiation. *Genes Dev.* **3**: 1639-46.

Keller, B., N. Sauer, and C. Lamb. 1988. Glycine-rich cell wall proteins in bean: gene structure and association of the protein with the vascular system. *EMBO J.* **7**: 3625.

Kieliszewski, M. and D.T.A. Lamport. 1987. Purification and partial characterization of a hydroxyproline-rich glycoprotein in a gramineous monocot, *Zea mays*. *Plant Physiol.* **85**: 823-827.

Kieliszewski, M., J.F. Leykam, and D.T.A. Lamport. 1990. Structure of the threonine-rich extensin from *Zea mays*. *Plant Physiol.* **92**: 316.

Kieliszewski, M.J., A. Kamyab, J.F. Leykam, and D.T.A. Lamport. 1992. A histidine-rich extensin from *Zea mays* is an arabinogalactan protein. *Plant Physiol.* **99**: 538-547.

Kieliszewski, M.J. and D.T. Lamport. 1994. Extensin: repetitive motifs, functional sites, post-translational codes, and phylogeny. *Plant J.* **5**: 157-172.

Kimura, S., W. Laosinchai, T. Itoh, X.J. Cui, C.R. Linder, and R.M. Brown. 1999. Immunogold labeling of rosette terminal cellulose-synthesizing complexes in the vascular plant *Vigna angularis*. *Plant Cell* **11**: 2075-2085.

Knox, J.P. 1995. The extracellular matrix in higher plants. 4. Developmentally regulated proteoglycans and glycoproteins of the plant cell surface. *FASEB J.* **9**: 1004-1012.

Kobe, B. and J. Deisenhofer. 1993. Crystal structure of porcine ribonuclease inhibitor, a protein with leucine-rich repeats. *Nature* **366**: 751-756.

Kobe, B. and J. Deisenhofer. 1994. The leucine-rich repeat: a versatile binding motif. *Trends Biochem. Sci.* **19**: 415-421.

Kobe, B. and J. Deisenhofer. 1995. A structural basis of the interactions between leucine-rich repeats and protein ligands. *Nature* **374**: 183-186.

Kobe, B. and J. Deisenhofer. 1996. Mechanism of ribonuclease inhibition by ribonuclease inhibitor protein based on the crystal structure of its complex with ribonuclease A. *J. Mol. Biol.* **264**: 1028-1043.

Kohorn, B.D. 2000. Plasma membrane-cell wall contacts. *Plant Physiol.* **124**: 31-38.

Kost, B., J. Mathur, and N.H. Chua. 1999. Cytoskeleton in plant development. *Curr. Opin. Plant Biol.* **2**: 462-470.

Krantz, D.E. and S.L. Zipursky. 1990. *Drosophila* chaoptin, a member of the leucine-rich repeat family, is a photoreceptor cell-specific adhesion molecule. *EMBO J.* **9**: 1969-1977.

Kresse, H., C. Liszio, E. Schonherr, and L.W. Fisher. 1997. Critical role of glutamate in a central leucine-rich repeat of decorin for interaction with type I collagen. *J. Biol. Chem.* **272**: 18404-18410.

Kreuger, M. and G.J. van Holst. 1996. Arabinogalactan proteins and plant differentiation. *Plant Mol. Biol.* **30**: 1077-1086.

Kropf, D., B. Kloareg, and R. Quatrano. 1988. Cell wall is required for fixation of the embryonic axis in *Fucus* zygotes. *Science* **239**: 187-190.

Leckie, F., B. Mattei, C. Capodicasa, A. Hemmings, L. Nuss, B. Aracri, G. De Lorenzo, and F. Cervone. 1999. The specificity of polygalacturonase-inhibiting protein (PGIP): a single amino acid substitution in the solvent-exposed beta- strand/beta-turn region of the leucine-rich repeats (LRRs) confers a new recognition capability. *EMBO J.* **18**: 2352-2363.

Lee, M.M. and J. Schiefelbein. 1999. WEREWOLF, a MYB-related protein in *Arabidopsis*, is a position- dependent regulator of epidermal cell patterning. *Cell* **99**: 473-483.

Lei, M. and R. Wu. 1991. A novel glycine-rich cell wall protein gene in rice. *Plant Mol. Biol.* **16**: 187-198.

Li, J.M. and J. Chory. 1997. A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. *Cell* **90**: 929-938.

Majewska-Sawka, A. and E.A. Nothnagel. 2000. The multiple roles of arabinogalactan proteins in plant development. *Plant Physiol.* **122**: 3-9.

Marfa, V., D. Gollin, S. Eberhard, D. Mohnen, A. Darvill, and P. Albersheim. 1991. Oligogalacturonides are able to induce flowers to form on tobacco explants. *Plant J.* **1**: 217-225.

Masucci, J.D. and J.W. Schiefelbein. 1994. The *rhd6* mutation of *Arabidopsis thaliana* alters root-hair initiation through an auxin-associated and ethylene-associated process. *Plant Physiol.* **106**: 1335-1346.

Masucci, J.D. and J.W. Schiefelbein. 1996. Hormones act downstream of *TTG* and *GL2* to promote root hair outgrowth during epidermis development in the *Arabidopsis* root. *Plant Cell* **8**: 1505-1517.

Mau, S.L., C.G. Chen, Z.Y. Pu, R.L. Moritz, R.J. Simpson, A. Bacic, and A.E. Clarke. 1995. Molecular cloning of cDNA encoding the protein backbones of arabinogalactan proteins from the filtrate of suspension-cultured cells of *Pyrus communis* and *Nicotiana alata*. *Plant J.* **8**: 269-281.

Merkouropoulos, G., D.C. Barnett, and A.H. Shirsat. 1999. The *Arabidopsis* extensin gene is developmentally regulated, is induced by wounding, methyl jasmonate, abscisic and salicylic acid, and codes for a protein with unusual motifs. *Planta* **208**: 212-219.

Miller, D.D., N.C.A. de Ruijter, T. Bisseling, and A.M.C. Emons. 1999. The role of actin in root hair morphogenesis: studies with lipochito-oligosaccharide as a growth stimulator and cytochalasin as an actin perturbing drug. *Plant J.* **17**: 141-154.

Nicol, F., I. His, A. Jauneau, S. Vernhettes, H. Canut, and H. Hofte. 1998. A plasma membrane-bound putative endo-1,4-beta-D-glucanase is required for normal wall assembly and cell elongation in *Arabidopsis*. *EMBO J.* **17**: 5563-5576.

Nielsen, H., J. Engelbrecht, S. Brunak, and G. von Heijne. 1997. A neural network method for identification of prokaryotic and eukaryotic peptides and prediction of their cleavage sites. *Int. J. Neural Sys.* **8**: 581-599.

Oxley, D. and A. Bacic. 1999. Structure of the glycosylphosphatidylinositol anchor of an arabinogalactan protein from *Pyrus communis* suspension-cultured cells. *Proc. Natl. Acad. Sci. USA* **96**: 14246-14251.

Papageorgiou, A.C., R. Shapiro, and K.R. Acharya. 1997. Molecular recognition of human angiogenin by placental ribonuclease inhibitor: an X-ray crystallographic study at 2.0 angstrom resolution. *EMBO J.* **16**: 5162-5177.

Parker, J.S., A.C. Cavell, L. Dolan, K. Roberts, and C.S. Grierson. 2000. Genetic interactions during root hair morphogenesis in *Arabidopsis*. *Plant Cell* **12**: 1961-1974.

Parniske, M., K.E. Hammond-Kosack, C. Golstein, C.M. Thomas, D.A. Jones, K. Harrison, B.B.H. Wulff, and J.D.G. Jones. 1997. Novel disease resistance specificities result from sequence exchange between tandemly repeated genes at the *Cf-4/9* locus of tomato. *Cell* **91**: 821-832.

Perrin, R.M., A.E. DeRocher, M. Bar-Peled, W.Q. Zeng, L. Norambuena, A. Orellana, N.V. Raikhel, and K. Keegstra. 1999. Xyloglucan fucosyltransferase, an enzyme involved in plant cell wall biosynthesis. *Science* **284**: 1976-1979.

Pierson, E.S., D.D. Miller, D.A. Callaham, A.M. Shipley, B.A. Rivers, M. Cresti, and P.K. Hepler. 1994. Pollen tube growth is coupled to the extracellular calcium ion flux and the intracellular calcium gradient: effect of BAPTA-type buffers and hypertonic media. *Plant Cell* **6**: 1815-1828.

Pierson, E.S., D.D. Miller, D.A. Callaham, J. van Aken, G. Hackett, and P.K. Hepler. 1996. Tip-localized calcium entry fluctuates during pollen tube growth. *Dev. Biol.* **174**: 160-173.

Qi, X.Y., B.X. Behrens, P.R. West, and A.J. Mort. 1995. Solubilization and partial characterization of extensin fragments from cell walls of cotton suspension cultures: evidence for a covalent cross-link between extensin and pectin. *Plant Physiol.* **108**: 1691-1701.

Reuzeau, C. and R.F. Pont-Lezica. 1995. Comparing plant and animal extracellular matrix-cytoskeleton connections: Are they alike? *Protoplasma* **186**: 113-121.

Rigas, S., G. Debrosses, K. Haralampidis, F. Vicente-Agullo, K.A. Feldmann, A. Grabov, L. Dolan, and P. Hatzopoulos. 2001. *TRH1* encodes a potassium transporter required for tip growth in *Arabidopsis* root hairs. *Plant Cell* **13**: 139-151.

Ringli, C., G. Hauf, and B. Keller. 2001. Hydrophobic interactions of the structural protein GRP1.8 in the cell wall of protoxylem elements. *Plant Physiol.* **125**: 673-682.

Roy, S.J., T.L. Holdaway-Clarke, G.R. Hackett, J.G. Kunkel, E.M. Lord, and P.K. Hepler. 1999. Uncoupling secretion and tip growth in lily pollen tubes: evidence for the role of calcium in exocytosis. *Plant J.* **19**: 379-386.

Rubinstein, A.L., A.H. Broadwater, K.B. Lowrey, and P.A. Bedinger. 1995a. *Pex1*, a pollen-specific gene with an extensin-like domain. *Proc. Natl. Acad. Sci. USA* **92**: 3086-3090.

Rubinstein, A.L., J. Marquez, M. Suarez Cervera, and P.A. Bedinger. 1995b. Extensin-like glycoproteins in the maize pollen tube wall. *Plant Cell* **7**: 2211-2225.

Ryan, C.A. and E.E. Farmer. 1991. Oligosaccharide signals in plants: a current assessment. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **42**: 651-674.

Ryser, U. and B. Keller. 1992. Ultrastructural localization of a bean glycine-rich protein in unlignified primary walls of protoxylem cells. *Plant Cell* **4**: 773-783.

Ryser, U., M. Schorderet, G.F. Zhao, D. Studer, K. Ruel, G. Hauf, and B. Keller. 1997. Structural cell-wall proteins in protoxylem development: Evidence for a repair process mediated by a glycine-rich protein. *Plant J.* **12**: 97-111.

Schiefelbein, J., M. Galway, J. Masucci, and S. Ford. 1993. Pollen tube and root hair tip growth is disrupted in a mutant of *Arabidopsis thaliana*. *Plant Physiol.* **103**: 979-985.

Schiefelbein, J., A. Shipley, and P. Rowse. 1992. Calcium influx at the tip of growing root hair cells of *Arabidopsis thaliana*. *Planta* **187**: 455-459.

Schiefelbein, J.W. 2000. Constructing a plant cell. The genetic control of root hair development. *Plant Physiol.* **124**: 1525-1531.

Schiefelbein, J.W. and C. Somerville. 1990. Genetic control of root hair development in *Arabidopsis thaliana*. *Plant Cell* **2**: 235-243.

Schnabelrauch, L.S., M. Kieliszewski, B.L. Upham, H. Alizedeh, and D.T. Lamport. 1996. Isolation of pI 4.6 extensin peroxidase from tomato cell suspension cultures and identification of Val-Tyr-Lys as putative intermolecular cross-link site. *Plant J.* **9**: 477-489.

Schneider, D.S., K.L. Hudson, T.Y. Lin, and K.V. Anderson. 1991. Dominant and recessive mutations define functional domains of Toll, a transmembrane protein required for dorsal-ventral polarity in the *Drosophila* embryo. *Genes Dev.* **5**: 797-807.

Schultz, C., P. Gilson, D. Oxley, J. Youl, and A. Bacic. 1998. GPI-anchors on arabinogalactan-proteins: implications for signaling in plants. *Trends Plant Sci.* **3**: 426-431.

Schultz, C.J., K.L. Johnson, G. Currie, and A. Bacic. 2000. The classical arabinogalactan protein gene family of *Arabidopsis*. *Plant Cell* **12**: 1751-1767.

Shirsat, A.H., A. Bell, J. Spence, and J.N. Harris. 1996. The *Brassica napus extA* extensin gene is expressed in regions of the plant subject to tensile stresses. *Planta* **199**: 618-624.

Showalter, A.M. 1993. Structure and function of plant cell wall proteins. *Plant Cell* **5**: 9-23.

Song, W.Y., G.L. Wang, L.L. Chen, H.S. Kim, L.Y. Pi, T. Holsten, J. Gardner, B. Wang, W.X. Zhai, L.H. Zhu, C. Fauquet, and P. Ronald. 1995. A receptor kinase-like protein encoded by the rice disease resistance gene, Xa21. *Science* **270**: 1804-1806.

Stafstrom, J. and L.A. Staehelin. 1986a. The role of carbohydrate in maintaining extensin in an extended conformation. *Plant Physiol.* **81**: 242-246.

Stafstrom, J.P. and L.A. Staehelin. 1986b. Cross-linking patterns in salt-extractable extensins from carrot cell walls. *Plant Physiol.* **81**: 234-241.

Steele, N.M., M.C. McCann, and K. Roberts. 1997. Pectin modification in cell walls of ripening tomatoes occurs in distinct domains. *Plant Physiol.* **114**: 373-381.

Svetek, J., M.P. Yadav, and E.A. Nothnagel. 1999. Presence of a glycosylphosphatidylinositol lipid anchor on rose arabinogalactan proteins. *J. Biol. Chem.* **274**: 14724-14733.

Tanimoto, M., K. Roberts, and L. Dolan. 1995. Ethylene is a positive regulator of root hair development in *Arabidopsis thaliana*. *Plant J.* **8**: 943-948.

Taylor, N.G., S. Laurie, and S.R. Turner. 2000. Multiple cellulose synthase catalytic subunits are required for cellulose synthesis in *Arabidopsis*. *Plant Cell* **12**: 2529-2539.

Taylor, N.G., W.R. Scheible, S. Cutler, C.R. Somerville, and S.R. Turner. 1999. The *irregular xylem3* locus of *Arabidopsis* encodes a cellulose synthase required for secondary cell wall synthesis. *Plant Cell* **11**: 769-779.

Templeton, M.D., R.A. Dixon, C.J. Lamb, and M.A. Lawton. 1990. Hydroxyproline-rich glycoprotein transcripts exhibit different spatial patterns of accumulation in compatible and incompatible interactions between *Phaseolus vulgaris* and *Colletotrichum lindemuthianum*. *Plant Physiol.* **94**: 1265-1269.

Thomas, C.M., M.S. Dixon, M. Parniske, C. Golstein, and J.D.G. Jones. 1998. Genetic and molecular analysis of tomato *Cf* genes for resistance to *Cladosporium fulvum*. *Philos. Trans. R. Soc. Lond. Ser. B-Biol. Sci.* **353**: 1413-1424.

Tierney, M.L., J. Wiechert, and D. Pluymers. 1988. analysis of the expression of extensin anf p33-related cell wall proteins in carrot and soybean. *Mol. Gen. Genet.* **211**: 393-399.

Torii, K.U. 2000. Receptor kinase activation and signal transduction in plants: an emerging picture. *Curr. Opin. Plant Biol.* **3**: 361-367.

Torii, K.U., N. Mitsukawa, T. Oosumi, Y. Matsuura, R. Yokoyama, R.F. Whittier, and Y. Komeda. 1996. The *Arabidopsis ERECTA* gene encodes a putative receptor protein kinase with extracellular leucine-rich repeats. *Plant Cell* **8**: 735-746.

Toubart, P., A. Desiderio, G. Salvi, F. Cervone, L. Daroda, and G. De Lorenzo. 1992. Cloning and characterization of the gene encoding the endopolygalacturonase-inhibiting protein (PGIP) of *Phaseolus vulgaris* L. *Plant J.* **2**: 367-373.

Trotochaud, A.E., S. Jeong, and S.E. Clark. 2000. CLAVATA3, a multimeric ligand for the CLAVATA1 receptor-kinase. *Science* **289**: 613-617.

van de Wiel, C., B. Scheres, H. Franssen, M.J. Vanlierop, A. Vanlammeren, A. Vankammen, and T. Bisseling. 1990. The early nodulin transcript *ENOD2* is located in the nodule parenchyma of pea and soybean root-nodules. *EMBO J.* **9**: 1-7.

van Holst, G.J. and J.E. Varner. 1984. Reinforced polyproline II conformation in a hydroxyproline-rich cell-wall glycoprotein from carrot root. *Plant Physiol.* **74**: 247-251.

Vision, T.J., D.G. Brown, and S.D. Tanksley. 2000. The origins of genomic duplications in *Arabidopsis. Science* **290**: 2114-2117.

Volkmann, D.B.F. 1999. Actin cytoskeleton in plants: from transport networks to signaling networks. *Microsc. Res. Tech.* **47**: 135-154.

Wada, T., T. Tachibana, Y. Shimura, and K. Okada. 1997. Epidermal cell differentiation in *Arabidopsis* determined by a Myb homolog, CPC. *Science* **277**: 1113-1116.

Walker, A.R., P.A. Davison, A.C. Bolognesi-Winfield, C.M. James, N. Srinivasan, T.L. Blundell, J.J. Esch, M.D. Marks, and J.C. Gray. 1999. The *TRANSPARENT TESTA GLABRA1* locus, which regulates trichome differentiation and anthocyanin biosynthesis in *Arabidopsis*, encodes a WD40 repeat protein. *Plant Cell* **11**: 1337-1349.

Wang, H.Y., S.K. Lockwood, M.F. Hoeltzel, and J.W. Schiefelbein. 1997. The *ROOT HAIR DEFECTIVE3* gene encodes an evolutionarily conserved protein with GTP-binding motifs and is required for regulated cell enlargement in *Arabidopsis*. *Genes Dev.* **11**: 799-811.

Weber, I.T., R.W. Harrison, and R.V. Iozzo. 1996. Model structure of decorin and implications for collagen fibrillogenesis. *J. Biol. Chem.* **271**: 31767-31770.

Willats, W.G.T. and J.P. Knox. 1996. A role for arabinogalactan-proteins in plant cell expansion: Evidence from studies on the interaction of beta-glucosyl Yariv reagent with seedlings of *Arabidopsis thaliana*. *Plant J.* **9**: 919-925.

Wilson, A.K., F.B. Pickett, J.C. Turner, and M. Estelle. 1990. A dominant mutation in *Arabidopsis* confers resistance to auxin, ethylene and abscisic-acid. *Mol. Gen. Genet.* **222**: 377-383.

Wilson, L.G. and J.C. Fry. 1986. Extensin: a major cell wall glycoprotein. *Plant Cell Environ.* **9**: 239-260.

Wisman, E., G.H. Cardon, P. Fransz, and H. Saedler. 1998. The behaviour of the autonomous maize transposable element *En/Spm* in *Arabidopsis thaliana* allows efficient mutagenesis. *Plant Mol. Biol.* **37**: 989-999.

Woessner, J.P. and U.W. Goodenough. 1994. Volvocine cell-walls and their constituent glycoproteins: an evolutionary perspective. *Protoplasma* **181**: 245-258.

Woessner, J.P., A.J. Molendijk, P. Vanegmond, F.M. Klis, U.W. Goodenough, and M.A. Haring. 1994. Domain conservation in several Volvocalean cell-wall proteins. *Plant Mol. Biol.* **26**: 947-960.

Wolfe, K.H., M.L. Gouy, Y.W. Yang, P.M. Sharp, and W.H. Li. 1989. Date of the monocot dicot divergence estimated from chloroplast DNA-sequence data. *Proc. Nat. Acad. Sci. USA.* **86**: 6201-6205.

Wulff, B.B.H., C.M. Thomas, M. Smoker, M. Grant, and J.D.G. Jones. 2001. Domain swapping and gene shuffling identify sequences required for induction of an Avr-dependent hypersensitive response by the tomato Cf-4 and Cf-9 proteins. *Plant Cell* **13**: 255-272.

Wyatt, S.E. and N.C. Carpita. 1993. The plant cytoskeleton-cell wall continuum. *Trends Cell Biol.* **3**: 413-417.

Wymer, C.L., T.N. Bibikova, and S. Gilroy. 1997. Cytoplasmic free calcium distributions during the development of root hairs of *Arabidopsis thaliana*. *Plant J.* **12**: 427-439.

Ye, Z.H., Y.R. Song, A. Marcus, and J.E. Varner. 1991. Comparative localization of three classes of cell wall proteins. *Plant J.* **1**: 175-83.

Ye, Z.H. and J.E. Varner. 1991. Tissue-specific expression of cell-wall proteins in developing soybean tissues. *Plant Cell* **3**: 23-37.

Youl, J.J., A. Bacic, and D. Oxley. 1998. Arabinogalactan-proteins from *Nicotiana alata* and *Pyrus communis* contain glycosylphosphatidylinositol membrane anchors. *Proc. Nat. Acad. Sci. USA.* **95**: 7921-7926.

Zhou, J., D. Rumeau, and A.M. Showalter. 1992. Isolation and characterization of two wound-regulated tomato extensin genes. *Plant Mol. Biol.* **20**: 5-17.

8. Appendix

8.1 Sequences

cDNA *LRX1* sequence, accession number: AY026364

```
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   1 ---------+---------+---------+---------+---------+---------+---------+---------+---------+
                                         M  L  F  P  P  L  R  S  L  F  L  F  T  L  L  L  S  S
     GTTTGTTTCTTACAAATCAAAGCAGACCACGACGATGAAAGCGACTTGGGCAGTGATATCAAAGTAGACAAACGTCTCAAGTTCGAGAAC
  91 ---------+---------+---------+---------+---------+---------+---------+---------+---------+
     V  C  F  L  Q  I  K  A  D  H  D  D  E  S  D  L  G  S  D  I  K  V  D  K  R  L  K  F  E  N
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 181 ---------+---------+---------+---------+---------+---------+---------+---------+---------+
     P  K  L  R  Q  A  Y  I  A  L  Q  S  W  K  K  A  I  F  S  D  P  F  N  F  T  A  N  W  N  G
     TCAGATGTTTGCTCCTACAATGGAATCTATTGTGCTCCTTCTCCTTCATACCCGAAAACCCGAGTCGTTGCTGGCATTGACCTCAACCAC
 271 ---------+---------+---------+---------+---------+---------+---------+---------+---------+
     S  D  V  C  S  Y  N  G  I  Y  C  A  P  S  P  S  Y  P  K  T  R  V  V  A  G  I  D  L  N  H
     GCTGACATGGCCGGTTACTTGGCTTCCGAGCTAGGTCTCCTCTCTGATCTCGCTCTGTTCCACATTAACTCAAACCGTTTCTGCGGTGAA
 361 ---------+---------+---------+---------+---------+---------+---------+---------+---------+
     A  D  M  A  G  Y  L  A  S  E  L  G  L  L  S  D  L  A  L  F  H  I  N  S  N  R  F  C  G  E
     GTCCCTCTCACGTTTAACCGCATGAAGCTTCTCTACGAGCTTGATCTTAGCAACAACCGATTCGTCGGTAAGTTCCCTAAGGTTGTCCTC
 451 ---------+---------+---------+---------+---------+---------+---------+---------+---------+
     V  P  L  T  F  N  R  M  K  L  L  Y  E  L  D  L  S  N  N  R  F  V  G  K  F  P  K  V  V  L
     TCTTTGCCTTCCCTTAAATTCTTGGATCTCCGCTACAACGAGTTTGAAGGCAAGATCCCATCAAAGCTCTTCGATAGAGAGCTTGACGCT
 541 ---------+---------+---------+---------+---------+---------+---------+---------+---------+
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     I  F  L  N  H  N  R  F  R  F  G  I  P  K  N  M  G  N  S  P  V  S  A  L  V  L  A  D  N  N
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 901 ---------+---------+---------+---------+---------+---------+---------+---------+---------+
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1081 ---------+---------+---------+---------+---------+---------+---------+---------+---------+
     C  I  T  G  L  A  R  Q  R  S  D  K  Q  C  S  S  L  L  A  R  P  V  D  C  S  K  F  G  C  Y
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1171 ---------+---------+---------+---------+---------+---------+---------+---------+---------+
     N  I  F  S  P  P  P  P  T  F  K  M  S  P  E  V  R  T  L  P  P  P  I  Y  V  Y  S  S  P  P
     CCACCACCGTCGTCAAAGATGTCCCCTACCGTCAGAGCATACTCTCCCCCACCACCACCTTCGTCCAAGATGTCACCCTCTGTCAGAGCA
1261 ---------+---------+---------+---------+---------+---------+---------+---------+---------+
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1351 ---------+---------+---------+---------+---------+---------+---------+---------+---------+
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     CCTTACGTATACTCATCTCCTCCACCGCCCTATGTGTACTCATCTCCTCCTCCACCGCCGTATGTGTATTCATCCCCTCCTCCACCGCCT
1441 ---------+---------+---------+---------+---------+---------+---------+---------+---------+
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     CCACCACCACCGTGTCCTGAATCATCTCCACCACCGCCTGTTGTATACTACGCACCCGTGACACAAAGCCCTCCACCACCATCACCGGTA
1621 ---------+---------+---------+---------+---------+---------+---------+---------+---------+
```

```
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1891 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
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1981 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
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2071 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
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2161 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
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    P  P  P  S  Y  Y
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2341 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
    ATATTATGTTCTTT
2431 ---------+---- 2444
```
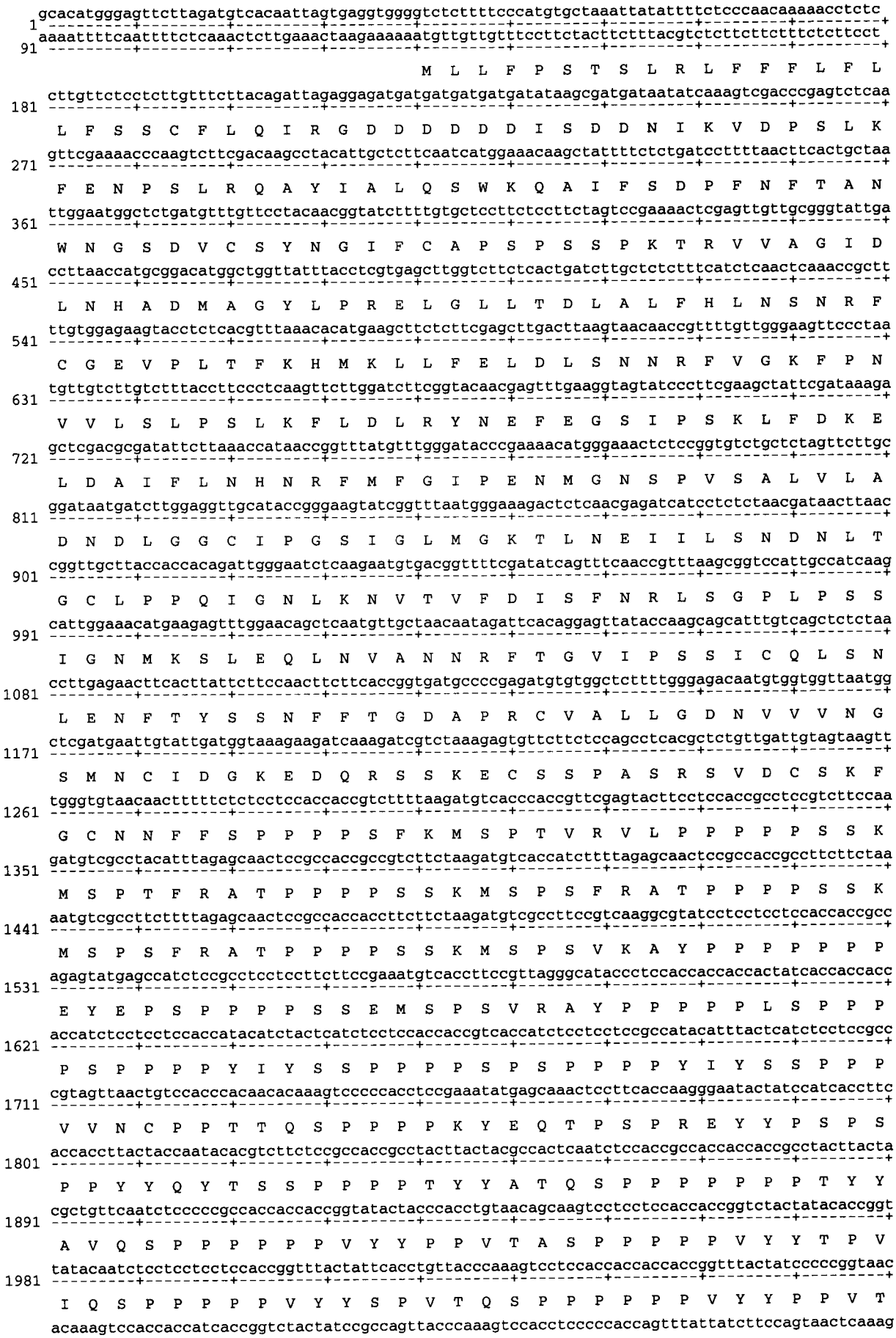

LRX2 sequence, accession number : AAF70841



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      G  C  N  D  S  P  S  N  D  H  H  Y  Q  T  P  T  P  P  S  L  P  P  P  Y  Y  E  D  T  P  L
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2431 ---------+---------+---------+---------+---------+---------+---------+---------+---------+
      P  P  I  R  G  V  S  Y  A  S  P  P  P  P  S  I  P  Y  Y  *
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     aaacagagaatatggagtatatagtttcac
2611 ---------+---------+---------+ 2640
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LRX3 Sequence, accession number: CAA19879

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  91 ---------+---------+---------+---------+---------+---------+---------+---------+---------+

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 181 ---------+---------+---------+---------+---------+---------+---------+---------+---------+

                                                    M  P  F  Y  K  Q  P  W  V  F  S  K  V
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 271 ---------+---------+---------+---------+---------+---------+---------+---------+---------+

      F  V  L  A  M  A  K  P  P  S  F  G  C  C  F  F  L  L  F  F  S  F  L  S  S  S  F  V  S  F
     TTGCCCTAACCGATACAGAAGCTGCCTTCATTGTGCAGCGACAACTTTTAACGTTACCAGACAACGGCGAACTTCCTGATGATATCGAAT
 361 ---------+---------+---------+---------+---------+---------+---------+---------+---------+

      A  L  T  D  T  E  A  A  F  I  V  Q  R  Q  L  L  T  L  P  D  N  G  E  L  P  D  D  I  E  Y
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 451 ---------+---------+---------+---------+---------+---------+---------+---------+---------+

      E  V  D  L  K  A  T  F  A  N  T  R  L  K  R  A  Y  I  A  L  Q  A  W  K  K  A  I  F  S  D
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 541 ---------+---------+---------+---------+---------+---------+---------+---------+---------+

      P  F  N  T  T  G  N  W  H  G  P  H  V  C  G  Y  T  G  V  V  C  A  P  A  L  D  D  S  D  V
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 631 ---------+---------+---------+---------+---------+---------+---------+---------+---------+

      T  V  V  A  G  V  D  L  N  G  A  D  I  A  G  H  L  P  A  E  L  G  L  M  T  D  V  A  M  F
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 721 ---------+---------+---------+---------+---------+---------+---------+---------+---------+

      H  L  N  S  N  R  F  C  G  I  I  P  K  S  F  E  K  L  K  L  M  H  E  F  D  V  S  N  N  R
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 811 ---------+---------+---------+---------+---------+---------+---------+---------+---------+

      F  V  G  P  F  P  N  V  V  L  S  W  P  D  V  K  Y  F  D  L  R  F  N  D  F  E  G  Q  V  P
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 901 ---------+---------+---------+---------+---------+---------+---------+---------+---------+

      P  E  L  F  K  K  E  L  D  A  I  F  L  N  D  N  R  F  T  S  V  I  P  E  S  L  G  E  S  P
     CGGCCTCGGTTGTGACTTTTGCTAACAATAAATTCACCGGATGTATCCCTAAGAGTATCGGAAACATGAAGAATCTCAACGAGATTGTCT
 991 ---------+---------+---------+---------+---------+---------+---------+---------+---------+

      A  S  V  V  T  F  A  N  N  K  F  T  G  C  I  P  K  S  I  G  N  M  K  N  L  N  E  I  V  F
     TTATGGACAATGATTTAGGAGGTTGTTTCCCATCTGAAATCGGAAAGTTATCGAATGTAACGGTTTTCGATGCAAGCAAGAACTCGTTCA
1081 ---------+---------+---------+---------+---------+---------+---------+---------+---------+

      M  D  N  D  L  G  G  C  F  P  S  E  I  G  K  L  S  N  V  T  V  F  D  A  S  K  N  S  F  I
     TAGGTCGTCTCCCGACTAGCTTTGTCGGGTTAACGAGCGTGGAGGAAATTGATATCTCCGGGAACAAACTCACCGGACTTGTACCGCATA
1171 ---------+---------+---------+---------+---------+---------+---------+---------+---------+

      G  R  L  P  T  S  F  V  G  L  T  S  V  E  E  I  D  I  S  G  N  K  L  T  G  L  V  P  H  N
     ACATTTGCCAGTTGCCTAATTTGGTGAACTTGACTTATTCTTACAATTACTTCAGTGGACAAGGAGGTTCTTGTGTTCCAGGTGGTAGTC
1261 ---------+---------+---------+---------+---------+---------+---------+---------+---------+

      I  C  Q  L  P  N  L  V  N  L  T  Y  S  Y  N  Y  F  S  G  Q  G  G  S  C  V  P  G  G  S  R
     GCAAGGAGATTGCGTTGGATGACACACGCAATTGCTTGGCTTCTCGACCTGAGCAACGATCCGCCCAAGAATGTGCGGTTGTGATCAACC
1351 ---------+---------+---------+---------+---------+---------+---------+---------+---------+

      K  E  I  A  L  D  D  T  R  N  C  L  A  S  R  P  E  Q  R  S  A  Q  E  C  A  V  V  I  N  R
     GCCCGGTTGATTGTAGCAAGGACAAGTGCGCTGGTGGCTCTTCTACTCCATCGAAACCATCACCGGTTCACAAACCAACGCCAGTTCCAA
1441 ---------+---------+---------+---------+---------+---------+---------+---------+---------+

      P  V  D  C  S  K  D  K  C  A  G  G  S  S  T  P  S  K  P  S  P  V  H  K  P  T  P  V  P  T
     CTACGCCGGTTCACAAACCAACACCAGTTCCAACTACGCCGGTTCAAAAACCATCGCCAGTTCCAACTACACCGGTTCAAAAACCATCGC
1531 ---------+---------+---------+---------+---------+---------+---------+---------+---------+

      T  P  V  H  K  P  T  P  V  P  T  T  P  V  Q  K  P  S  P  V  P  T  T  P  V  Q  K  P  S  P
     CAGTTCCAACAACCCCTGTTCACGAACCATCGCCAGTTTTAGCTACGCCGGTTGACAAACCATCGCCAGTTCCATCTCGGCCGGTTCAAA
1621 ---------+---------+---------+---------+---------+---------+---------+---------+---------+

      V  P  T  T  P  V  H  E  P  S  P  V  L  A  T  P  V  D  K  P  S  P  V  P  S  R  P  V  Q  K
     AACCACAACCACCAAAAGAGTCACCTCAACCAGATGATCCTTACGATCAATCTCCAGTGACGAAAAGGCGCAGCCCTCCTCCGGCTCCGG
1711 ---------+---------+---------+---------+---------+---------+---------+---------+---------+

      P  Q  P  P  K  E  S  P  Q  P  D  D  P  Y  D  Q  S  P  V  T  K  R  R  S  P  P  P  A  P  V
     TCAACTCTCCACCACCACCAGTTTACTCCCCACCACCTCCTCCTCCACCCGTTCACTCTCCCCCGCCGCCAGTCCACTCTCCTCCACCAC
1801 ---------+---------+---------+---------+---------+---------+---------+---------+---------+

      N  S  P  P  P  P  V  Y  S  P  P  P  P  P  P  P  V  H  S  P  P  P  P  V  H  S  P  P  P  P
     CACCGGTGTATTCGCCTCCGCCCCCACCCCCACCTGTCCACTCTCCTCCACCGCCTGTCTTTTCACCACCACCTCCAGTCTATTCCCCGC
1891 ---------+---------+---------+---------+---------+---------+---------+---------+---------+

      P  V  Y  S  P  P  P  P  P  P  P  V  H  S  P  P  P  P  V  F  S  P  P  P  P  V  Y  S  P  P
     CTCCCCCAGTCCACTCACCGCCTCCCCCAGTCCATTCGCCGCCGCCGCCAGCACCTGTTCACTCACCGCCTCCACCGGTTCATTCCCCAC
1981 ---------+---------+---------+---------+---------+---------+---------+---------+---------+

      P  P  V  H  S  P  P  P  P  V  H  S  P  P  P  P  A  P  V  H  S  P  P  P  P  V  H  S  P  P
     CGCCACCACCACCTGTTTATTCACCGCCACCACCGGTCTTTTCTCCACCGCCGTCGCAATCTCCACCAGTAGTCTACTCTCCTCCGCCAA
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2071 ---------+---------+---------+---------+---------+---------+---------+---------+---------+
       P  P  P  P  V  Y  S  P  P  P  P  V  F  S  P  P  P  S  Q  S  P  P  V  V  Y  S  P  P  P  R
     GACCGCCCAAGATCAACTCCCCACCAGTCCAATCACCACCGCCAGCACCGGTGGAAAAGAAAGAGACCCCTCCGGCTCATGCACCAGCTC
2161 ---------+---------+---------+---------+---------+---------+---------+---------+---------+
       P  P  K  I  N  S  P  P  V  Q  S  P  P  P  A  P  V  E  K  K  E  T  P  P  A  H  A  P  A  P
     CAAGTGACGATGAATTCATCATACCACCGTTCATCGGCCACCAATACGCATCACCACCACCGCCAATGTTCGCAGGCTACTAATGATCTC
2251 ---------+---------+---------+---------+---------+---------+---------+---------+---------+
       S  D  D  E  F  I  I  P  P  F  I  G  H  Q  Y  A  S  P  P  P  P  M  F  A  G  Y  *
     AATCGAAGAGAGAAGAATTCTATATATGAGTAGCAACTGCATCGTCTTGGACTAATTGTAGTAAAACTATGAAAAAGAACAAAATTCGAG
2341 ---------+---------+---------+---------+---------+---------+---------+---------+---------+
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2431 ---------+---------+---------+ 2460
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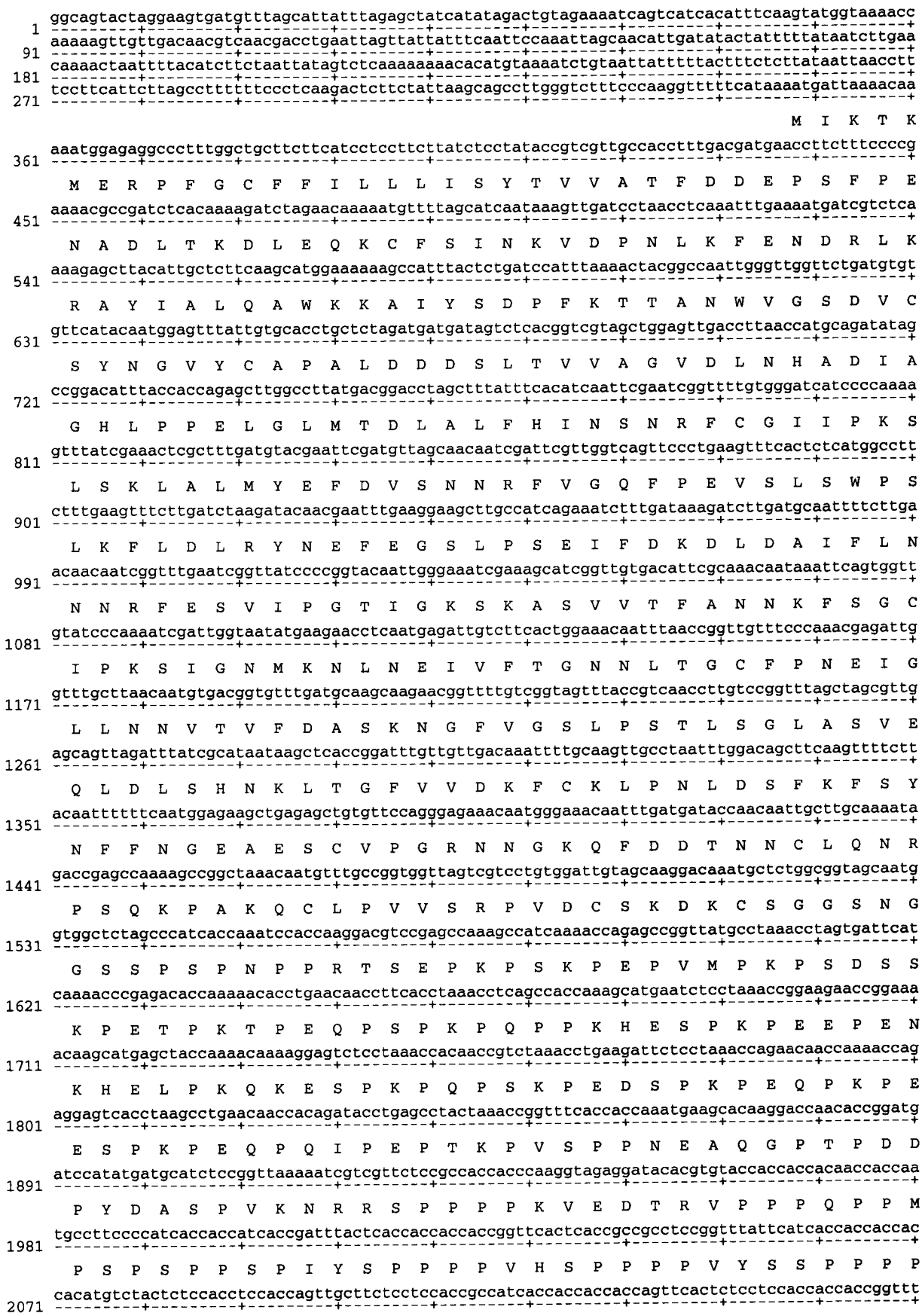
LRX4 sequence, accession number: AAD43152



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2161 tctctccaccaccaccagttttctctccaccaccaccatcaccagtatactcaccaccgcctccaagccactcaccaccaccaccagtat
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  S  P  P  P  P  V  F  S  P  P  P  P  S  P  V  Y  S  P  P  P  P  S  H  S  P  P  P  P  V  Y

2251 attctccgcctccaccaactttctcaccaccaccaacacataatacaaaccaaccaccaatgggagctccaacaccgactcaagcaccaa
---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
  S  P  P  P  P  T  F  S  P  P  P  T  H  N  T  N  Q  P  P  M  G  A  P  T  P  T  Q  A  P  T

2341 caccatcctctgaaacaacccaagtgccaactccatcatcagaatcagatcaatcacaaattttgtcaccagttcaagctccaacaccgg
---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
  P  S  S  E  T  T  Q  V  P  T  P  S  S  E  S  D  Q  S  Q  I  L  S  P  V  Q  A  P  T  P  V

2431 ttcaatcctcaaccccatcctctgaaccaacccaagtgccaactccatcatcatcagaatcatatcaagcaccaaatttgtcaccagttc
---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
  Q  S  S  T  P  S  S  E  P  T  Q  V  P  T  P  S  S  S  E  S  Y  Q  A  P  N  L  S  P  V  Q

2521 aagctccaacaccggttcaagccccaaccacatcctctgaaacatctcaagtaccaactccatcatcagaatcaaaccagtcaccaagtc
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  A  P  T  P  V  Q  A  P  T  T  S  S  E  T  S  Q  V  P  T  P  S  S  E  S  N  Q  S  P  S  Q

2611 aagctccaacaccaattcttgaaccggttcatgccccaacaccaaactctaaaccggtccaatcaccaacaccatcaagcgaaccggtct
---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
  A  P  T  P  I  L  E  P  V  H  A  P  T  P  N  S  K  P  V  Q  S  P  T  P  S  S  E  P  V  S

2701 catcaccagaacaatcagaagaagttgaagcaccagaaccaacacccgtcaatccatcatcagtaccatcatcttctccttccactgaca
---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
  S  P  E  Q  S  E  E  V  E  A  P  E  P  T  P  V  N  P  S  S  V  P  S  S  S  P  S  T  D  T

2791 catctattccaccaccggaaaacaacgacgatgatgatgatggggatttcgtcctaccaccacacattggattccaatatgcatcgccac
---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
  S  I  P  P  P  E  N  N  D  D  D  D  D  G  D  F  V  L  P  P  H  I  G  F  Q  Y  A  S  P  P

2881 caccaccaatgttccaaggatattaaaaccaatgtagtagtgacagtgttacccatttttaacatttctgtccatttttttccatgtgaat
---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
  P  P  M  F  Q  G  Y  *

2971 tatttgtatcaaacgaaaaagatattacaaagatgaaaaattaaaatgccaataattatatagtagattatctttcagcagatttttttt
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---------+---------+ 3080
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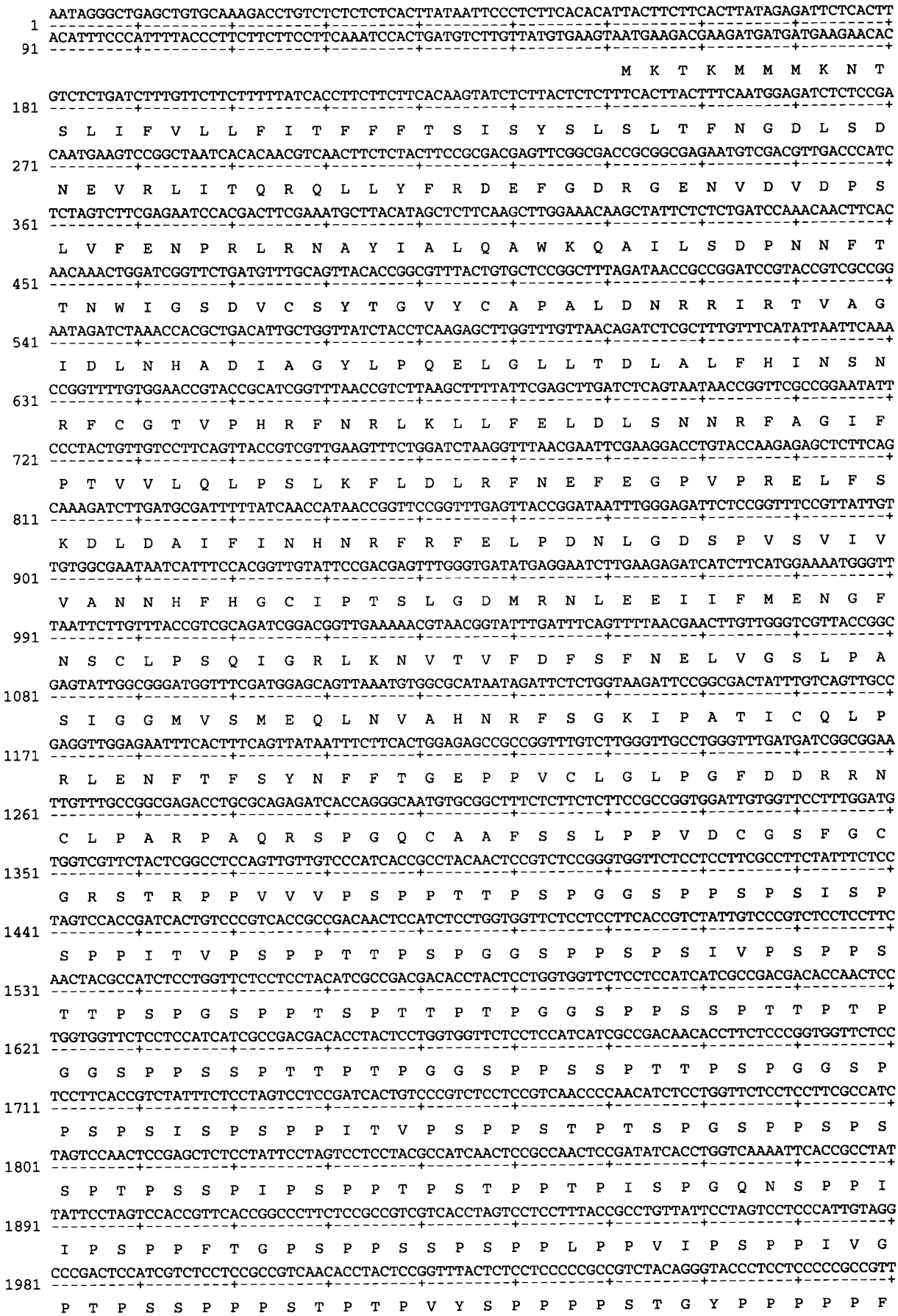
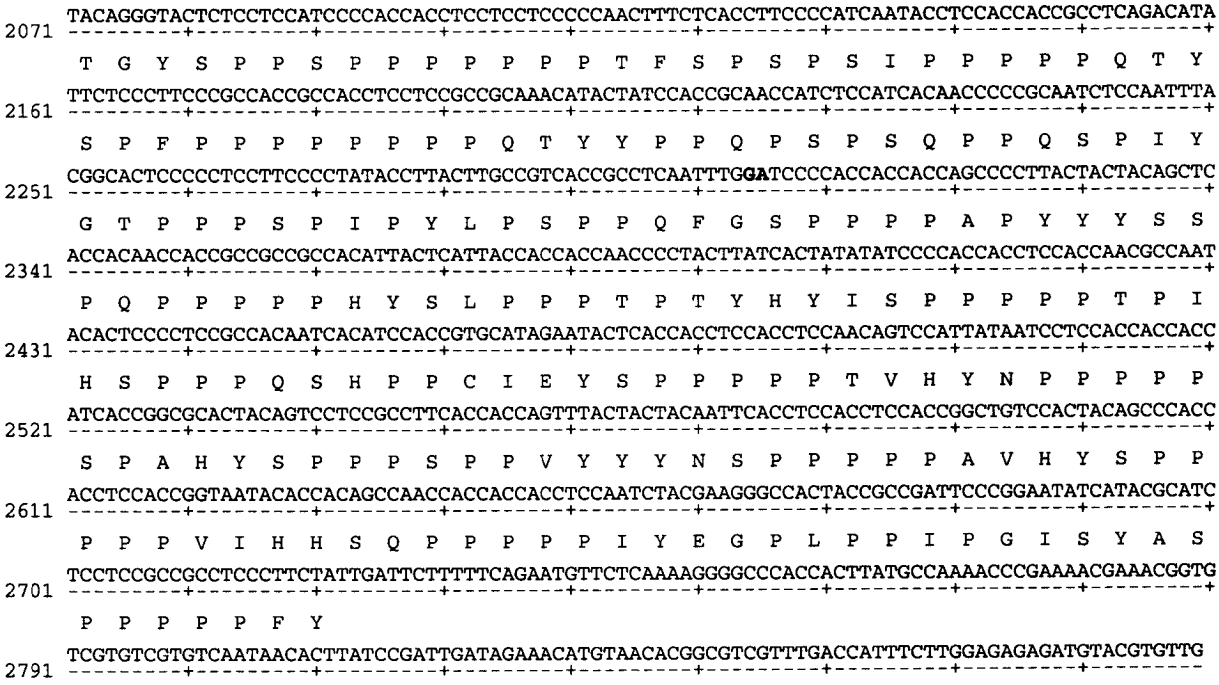
LRX5 sequence, accession number: CAB37452

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T G Y S P P S P P P P P P P T F S P S P S I P P P P P Q T Y
2161
TTCTCCCTTCCCGCCACCGCCACCTCCTCCGCCGCAAACATACTATCCACCGCAACCATCTCCATCACAACCCCCGCAATCTCCAATTTA
S P F P P P P P P P P Q T Y Y P P Q P S P S Q P P Q S P I Y
2251
CGGCACTCCCCCTCCTTCCCCTATACCTTACTTGCCGTCACCGCCTCAATTTGGATCCCCACCACCACCAGCCCCTTACTACTACAGCTC
G T P P P S P I P Y L P S P P Q F G S P P P P A P Y Y Y S S
2341
ACCACAACCACCGCCGCCGCCACATTACTCATTACCACCACCAACCCCTACTTATCACTATATATCCCCACCACCTCCACCAACGCCAAT
P Q P P P P P H Y S L P P P T P T Y H Y I S P P P P P T P I
2431
ACACTCCCCTCCGCCACAATCACATCCACCGTGCATAGAATACTCACCACCTCCACCTCCAACAGTCCATTATAATCCTCCACCACCACC
H S P P P Q S H P P C I E Y S P P P P P T V H Y N P P P P P
2521
ATCACCGGCGCACTACAGTCCTCCGCCTTCACCACCAGTTTACTACTACAATTCACCTCCACCTCCACCGGCTGTCCACTACAGCCCACC
S P A H Y S P P P S P P V Y Y Y N S P P P P P A V H Y S P P
2611
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P P P V I H H S Q P P P P P I Y E G P L P P I P G I S Y A S
2701
TCCTCCGCCGCCTCCCTTCTATTGATTCTTTTTCAGAATGTTCTCAAAAGGGGCCCACCACTTATGCCAAAACCCGAAAACGAAACGGTG
P P P P P F Y
2791
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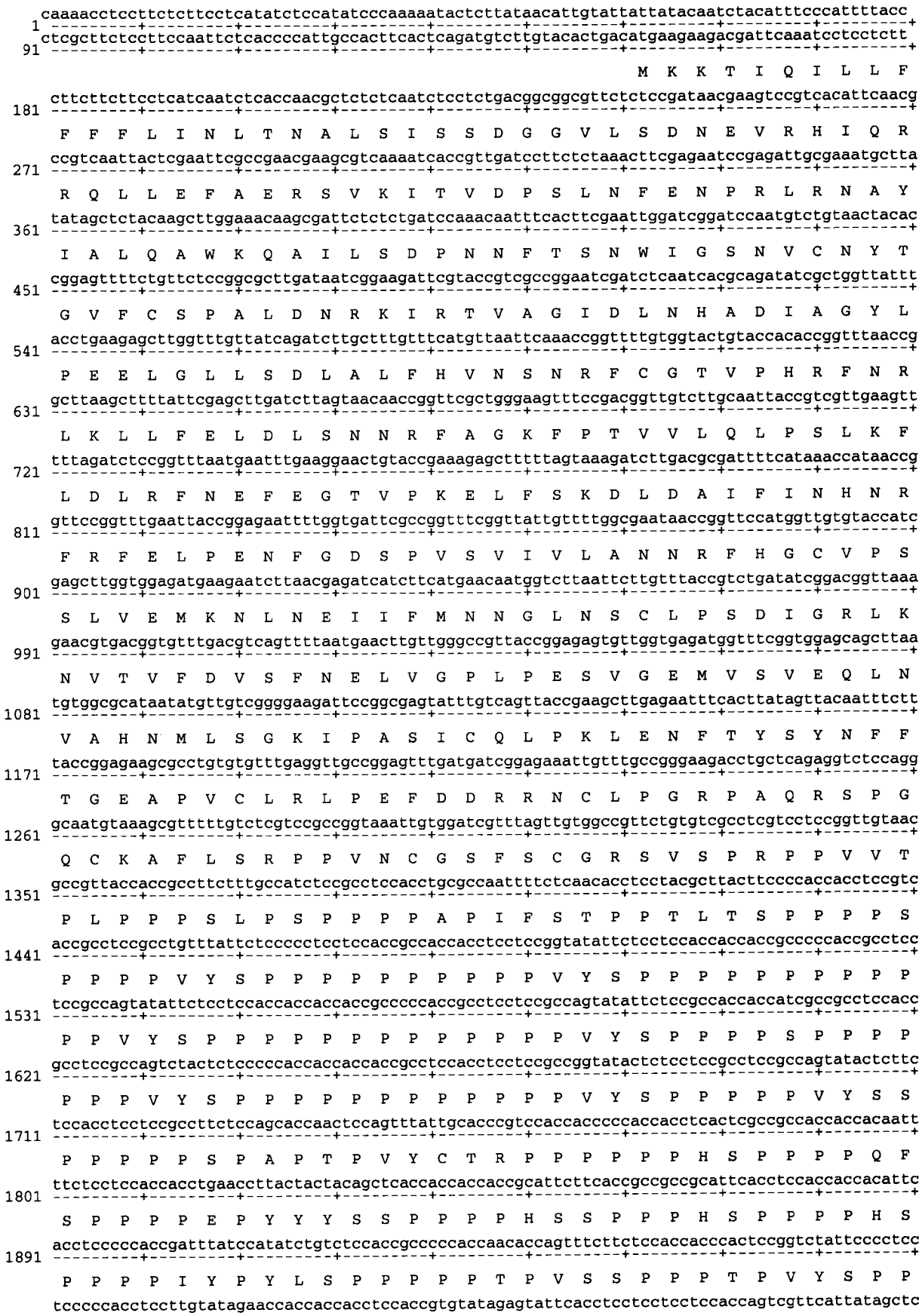
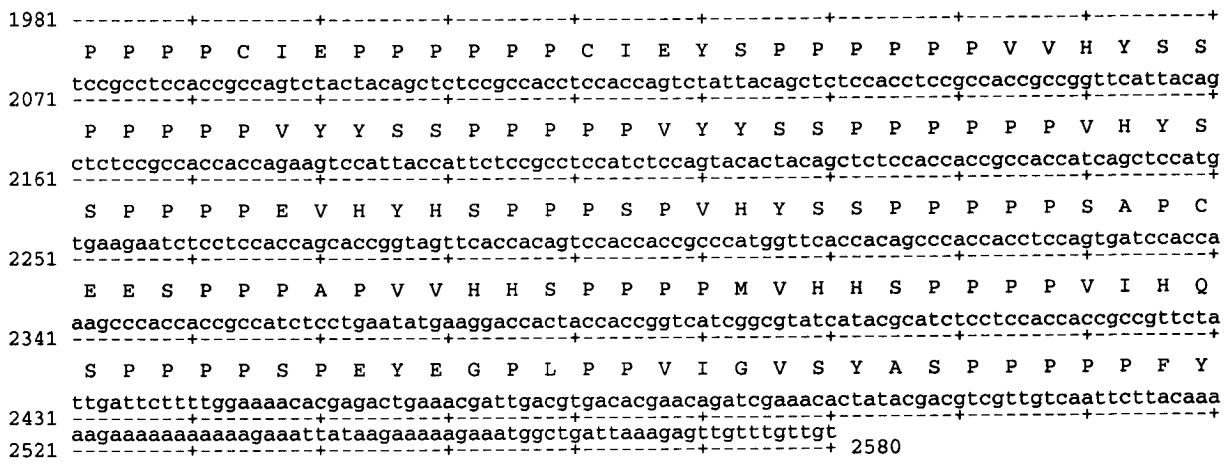
LRX6 sequence, accession number: CAB40769

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2071
P P P P P V Y Y S S P P P P P V Y Y S S P P P P P P V H Y S
ctctccgccaccaccagaagtccattaccattctccgcctccatctccagtacactacagctctccaccaccgccaccatcagctccatg
2161
S P P P P E V H Y H S P P P S P V H Y S S P P P P P S A P C
tgaagaatctcctccaccagcaccggtagttcaccacagtccaccaccgcccatggttcaccacagcccaccacctccagtgatccacca
2251
E E S P P P A P V V H H S P P P P M V H H S P P P P V I H Q
aagcccaccaccgccatctcctgaatatgaaggaccactaccaccggtcatcggcgtatcatacgcatctcctccaccaccgccgttcta
2341
S P P P P S P E Y E G P L P P V I G V S Y A S P P P P P F Y
ttgattcttttggaaaacacgagactgaaacgattgacgtgacacgaacagatcgaaacactatacgacgtcgttgtcaattcttacaaa
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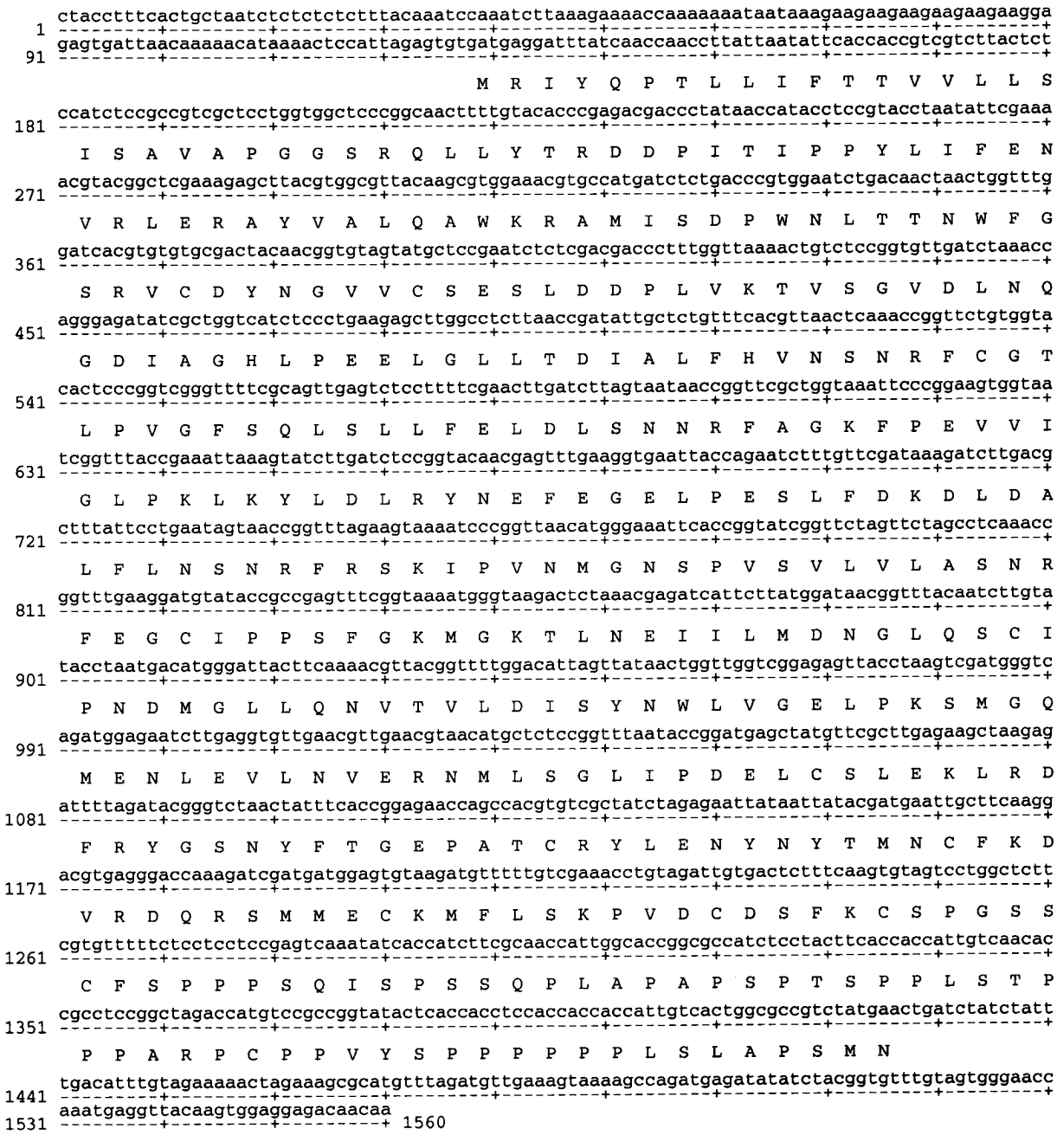
2521
2580

LRX7 sequence, accession number: AC006601



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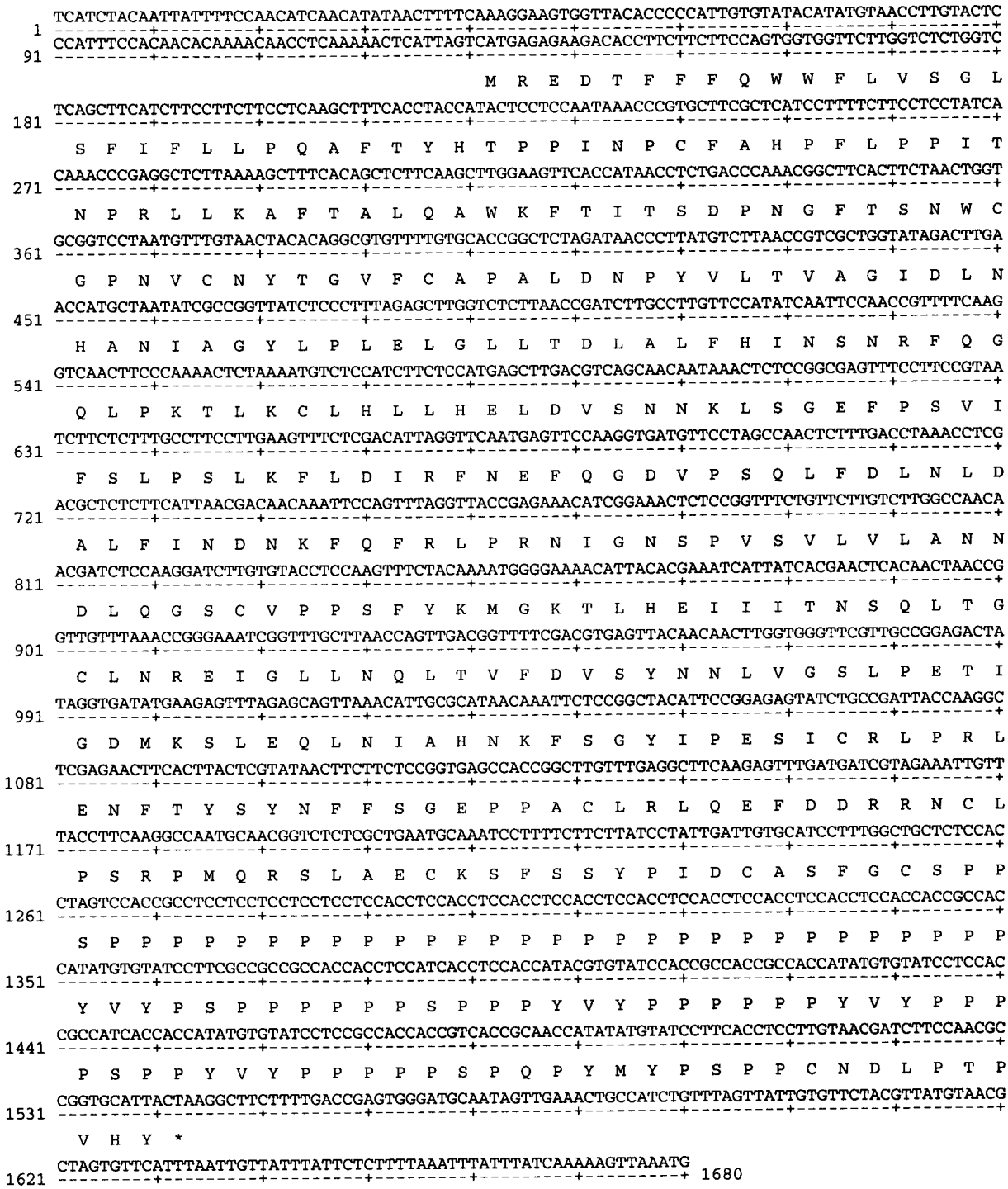

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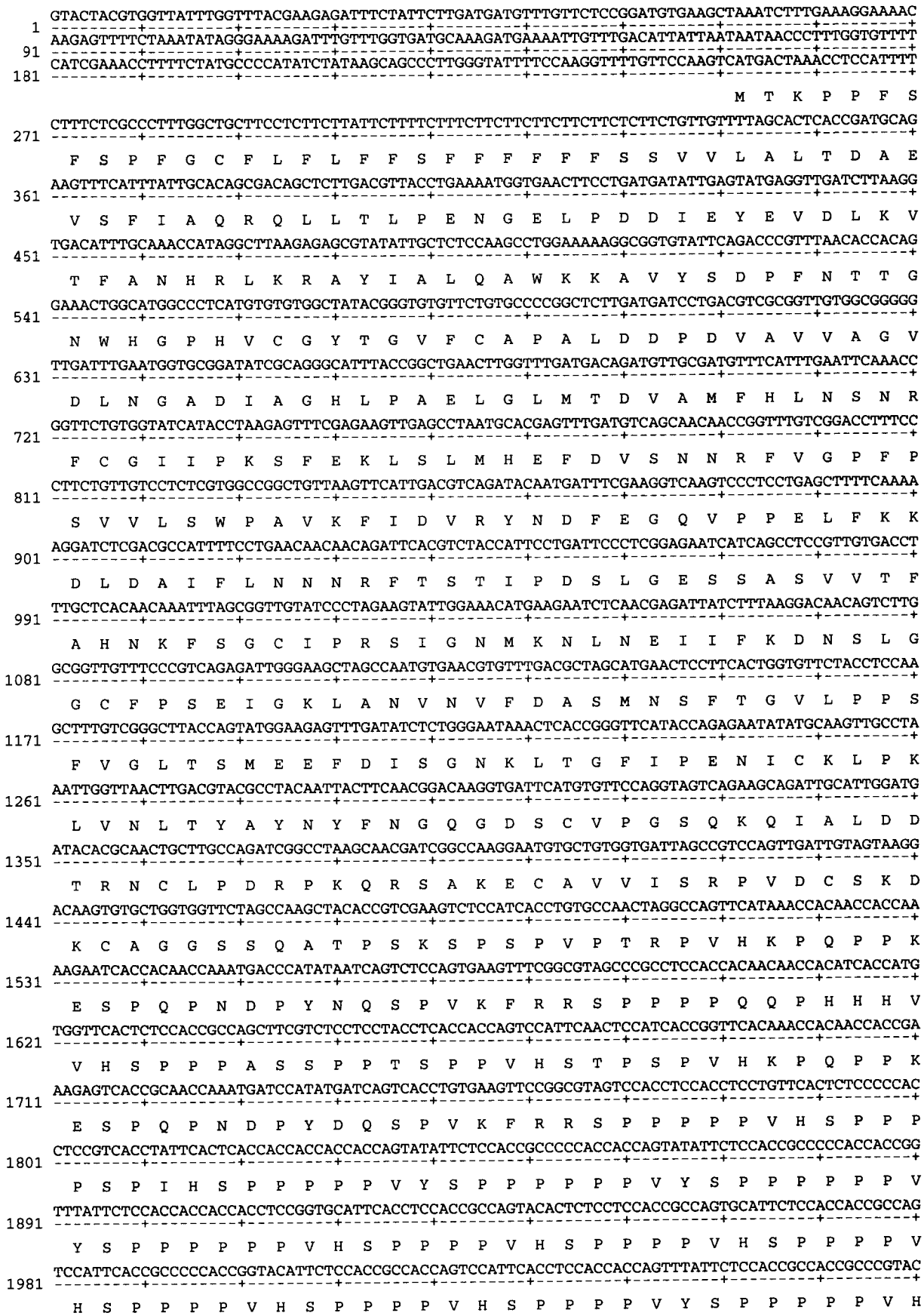

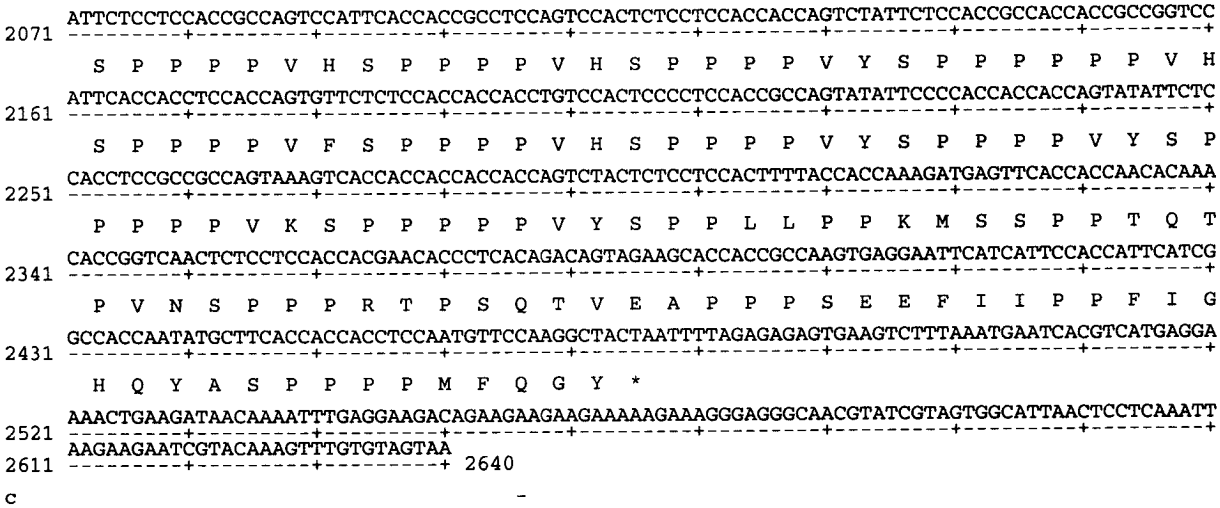
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2161
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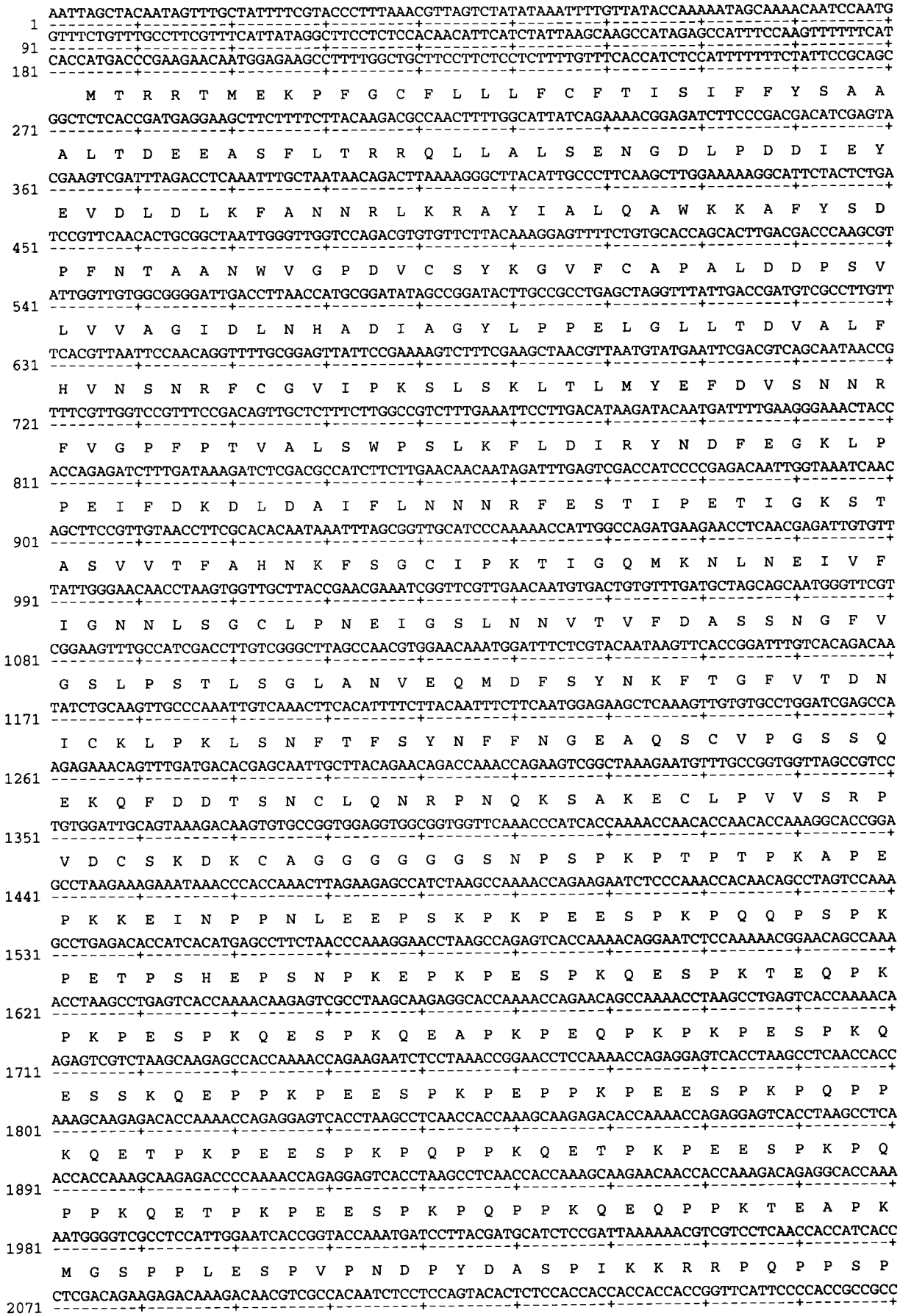
2251
P P P P V K S P P P P P V Y S P P L L P P K M S S P P T Q T
2341
P V N S P P P R T P S Q T V E A P P P S E E F I I P P F I G
2431
H Q Y A S P P P P M F Q G Y *
2521
2611
2640
c

LRX10 sequence, accession number: BAB01698



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2161 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
     V  F  S  P  P  P  P  M  H  S  P  P  P  P  V  Y  S  P  P  P  P  V  H  S  P  P  P  P  P  V
     CCACTCTCCTCCACCACCAGTACATTCACCACCACCACCAGTCCATTCTCCTCCTCCACCGGTCCATTCACCACCACCACCAGTCCACTC
2251 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
     H  S  P  P  P  P  V  H  S  P  P  P  P  V  H  S  P  P  P  P  V  H  S  P  P  P  P  V  H  S
     TCCTCCTCCACCAGTCCACTCTCCTCCTCCACCGGTCCAGTCACCTCCACCACCACCAGTTTTCTCTCCACCACCGCCTGCACCAATCTA
2341 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
     P  P  P  P  V  H  S  P  P  P  P  V  Q  S  P  P  P  P  P  V  F  S  P  P  P  P  A  P  I  Y
     CTCACCTCCTCCTCCACCAGTCCACTCCCCGCCTCCACCGGTCCACTCTCCACCACCGCCACCAGTCCACTCACCACCTCCTCCTGTTCA
2431 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
     S  P  P  P  P  P  V  H  S  P  P  P  P  V  H  S  P  P  P  P  P  V  H  S  P  P  P  P  V  H
     CTCACCGCCTCCTCCAGTTCACTCCCCGCCGCCTCCAGTCCATTCTCCACCACCACCAGTCCACTCTCCACCACCTCCGTCACCTATCTA
2521 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
     S  P  P  P  P  V  H  S  P  P  P  P  V  H  S  P  P  P  P  V  H  S  P  P  P  P  S  P  I  Y
     CTCTCCTCCTCCTCCAGTTTTCTCACCGCCACCGAAACCTGTAACGCCTCTACCACCAGCAACATCGCCAATGGCAAACGCTCCAACACC
2611 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
     S  P  P  P  P  V  F  S  P  P  P  K  P  V  T  P  L  P  P  A  T  S  P  M  A  N  A  P  T  P
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2701 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
     S  S  S  E  S  G  E  I  S  T  P  V  Q  A  P  T  P  D  S  E  D  I  E  A  P  S  D  S  N  H
     TTCACCAGTGTTCAAATCAAGTCCAGCTCCATCACCAGATTCCGAGCCAGAAGTTGAAGCACCAGTACCATCAAGCGAACCAGAAGTTGA
2791 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
     S  P  V  F  K  S  S  P  A  P  S  P  D  S  E  P  E  V  E  A  P  V  P  S  S  E  P  E  V  E
     AGCACCAAAACAATCAGAAGCCACCCCTTCGTCATCACCGCCATCTTCAAATCCATCACCAGATGTAACTGCACCACCATCGGAAGACAA
2881 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
     A  P  K  Q  S  E  A  T  P  S  S  S  P  P  S  S  N  P  S  P  D  V  T  A  P  P  S  E  D  N
     CGACGACGGCGATAATTTCATCCTCCCACCAAACATTGGACACCAATATGCATCGCCACCACCACCGATGTTCCCAGGCTACTGAACCAT
2971 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
     D  D  G  D  N  F  I  L  P  P  N  I  G  H  Q  Y  A  S  P  P  P  P  M  F  P  G  Y  *
     ACATTTTTAATATTTTACATTTTGGTCCAACATTTTTCCGGTTAACGCAATTTGTAACAAACAAAAAAAAAGATACATTTATAATTTGAC
3061 ---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+
     CATTTGTAATCTGTAAGTAGATGATTTTTA
3151 ---------+---------+---------+ 3180
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LRX11 sequence, accession number: BAB01951

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91   CCCTTCTTCCTCCTTCGAATTTTCAAACCTCTACCACTCCTCACGCAGATGTATCGTATCGTGAAATGAAGAACAACACCACTCAATCTC
     L  L  P  P  S  N  F  Q  T  S  T  T  P  H  A  D  V  S  Y  R  E  M  K  N  N  T  T  Q  S  L
181  TCCTTCTCCTTCTCCTCTTCTTCTTCTTCTTTGAAATCTCTCATTCTCTCTCAATCTCATCCAATGCTCCTCTCTCCGACACCGAAG
     L  L  L  L  L  F  F  F  F  F  F  E  I  S  H  S  L  S  I  S  S  N  A  P  L  S  D  T  E  V
271  TCAGATTCATCCAACGCCGTCAACTTCTCTACTACCGCGACGAGTTCGGTGACCGTGGTGAGAATGTCACAGTAGATCCATCACTAATCT
     R  F  I  Q  R  R  Q  L  L  Y  Y  R  D  E  F  G  D  R  G  E  N  V  T  V  D  P  S  L  I  F
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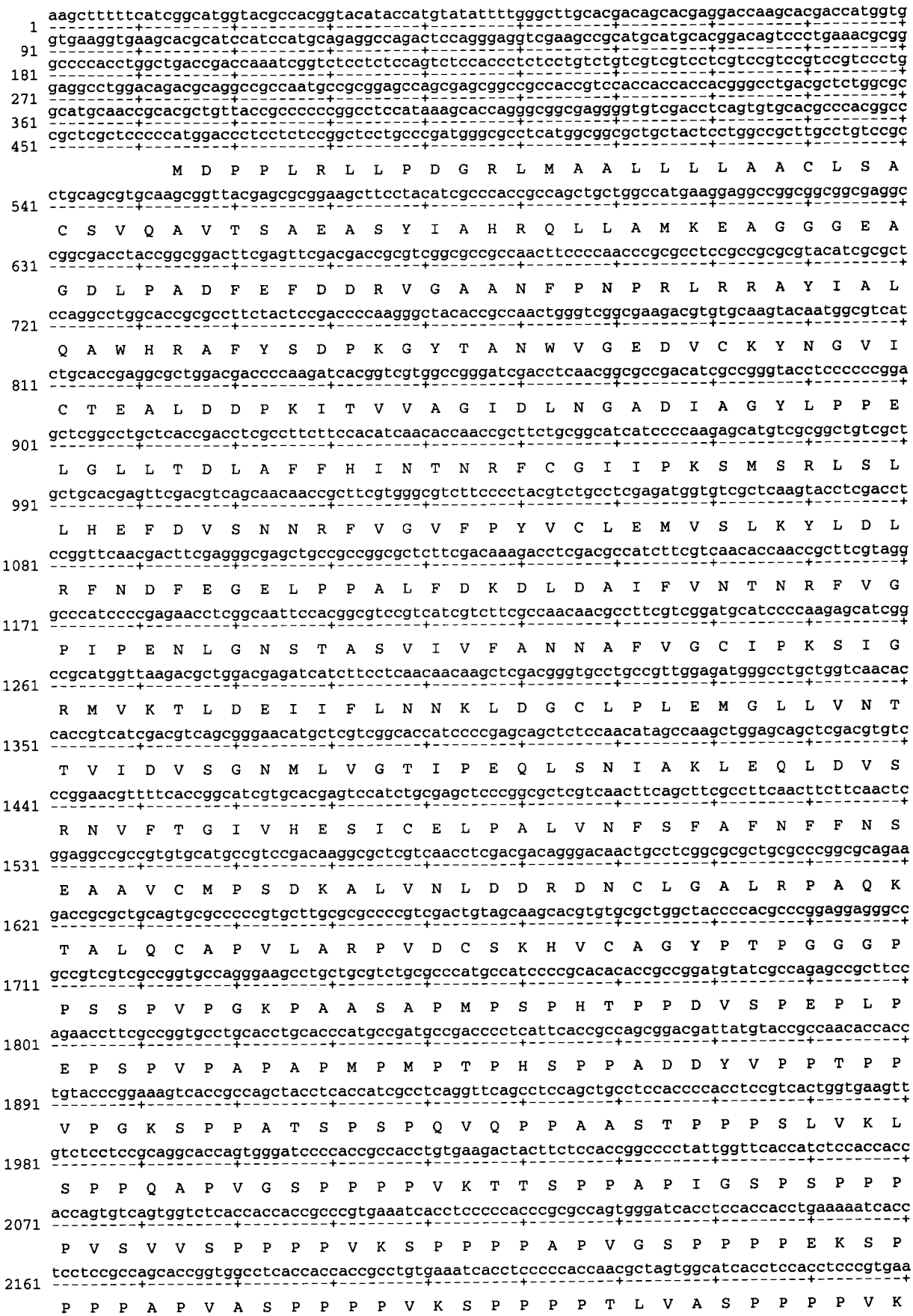

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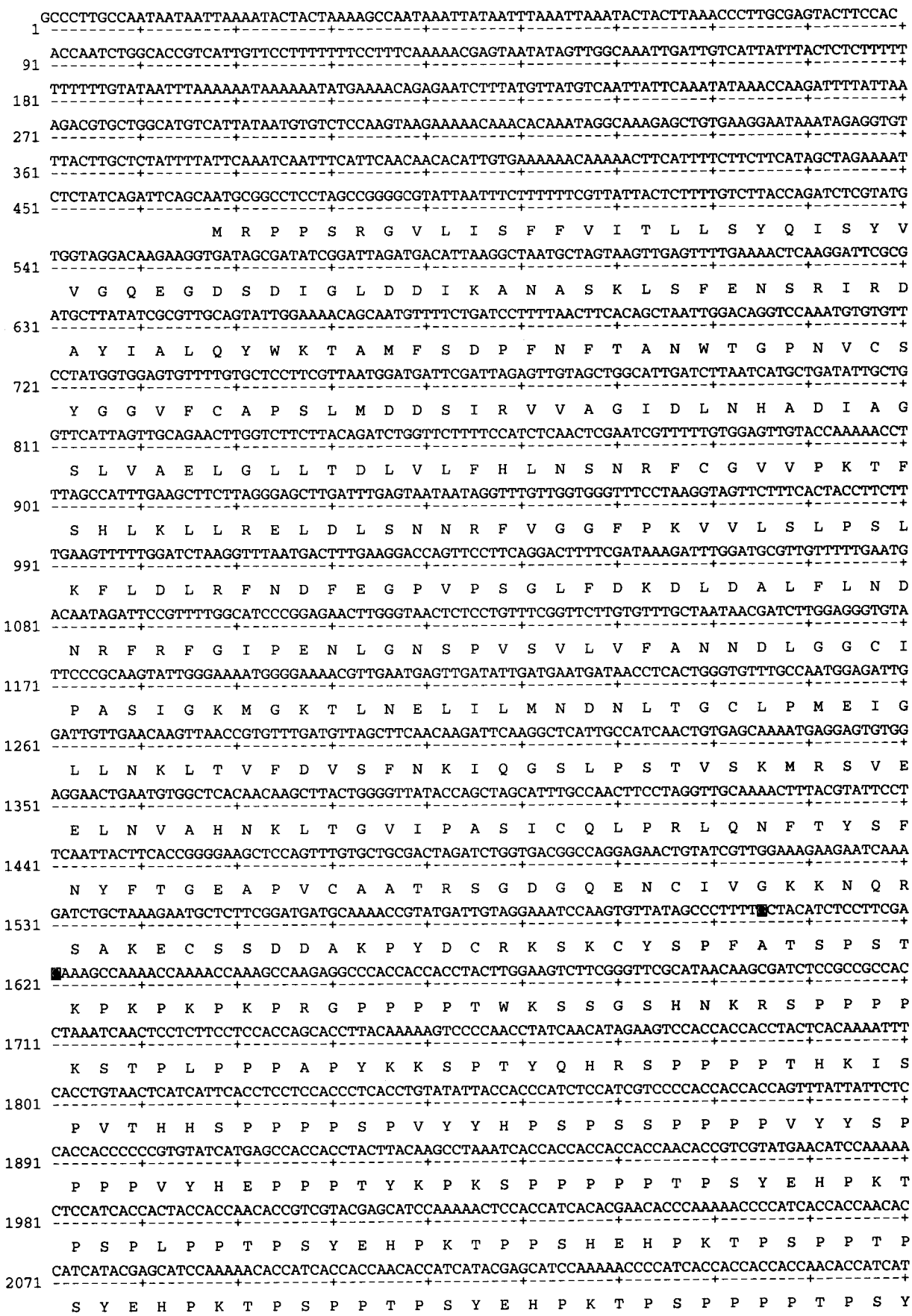

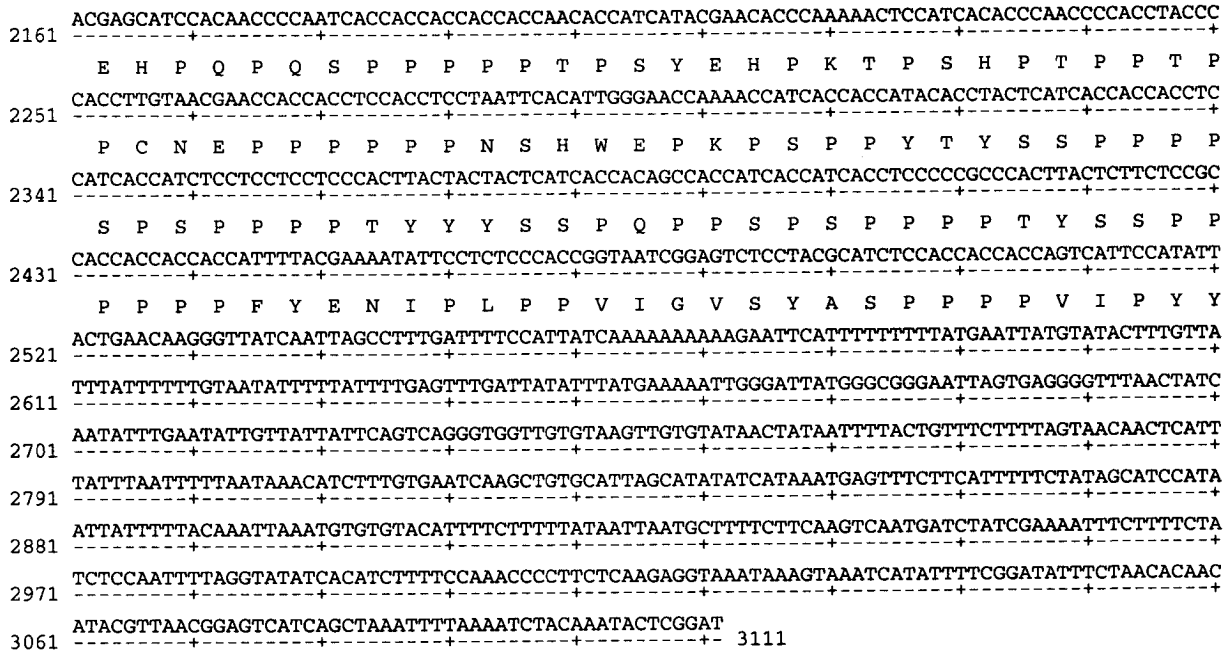
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8.2 Abbreviations

AGP	arabinogalactan protein
AVG	L-α-(2-amino-ethoxyvinyl)glycine
BHLH	basic helix-loop helix
bp	base pair
BSA	bovine serum albumine
CAM	cell-adhesion molecule
CaMV	cauliflower mosaic virus
DTT	1.4-dithio-DL-threitol
ECM	extracellular matrix
EDTA	disodium ethylene diamine tetraacetate
GRP	glycine-rich protein
HEPES	4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid
HRGP	hydroxyproline-rich glycoprotein
IgG	immunoglobuline G
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase
kDa	kilodalton
LRR	leucine-rich repeat
LRX	leucine-rich repeat/extensin
MES	2-(N-morpholino) ethanesulfonic acid
MS	Murashige and Skoog
O.D.	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PRP	proline-rich protein
PVDF	polyvinyl difluoride
RFLP	restriction fragment length polymorphism
RLK	receptor-like kinase
SDS	sodium dodecyl sulfate
SSC	sodium salt citrate
TBS	tris buffer saline
TRIS	tris-hydroxymethyl-aminomethane

8.3 Acknowledgements

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elle m'a fait l'heureux papa, ces années, si interessantes qu'elles fussent, auraient eu tellement moins de charme.

8.4 Curriculum vitae

June 12, 1970	Born in Lausanne (VD), Switzerland.
1978-1981	Primary school in Lausanne and Cugy (VD).
1981-1986	Secondary school in Lausanne.
1986-1989	High school in Pully (VD).
1989	High school graduation (Maturité type B, Bacchalauréat latin-math).
1989-1993	Biology studies. University of Lausanne.
1993	University graduation (licence en Biologie).
1993-1997	Research assistant, Institute of Plant Physiology, University of Lausanne.
1997	Diploma thesis in Biology,