

# Pleiotropic control of glucose and hormone responses by PRL1, a nuclear WD protein, in *Arabidopsis*

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**The *prl1* mutation localized by T-DNA tagging on *Arabidopsis* chromosome 4-44 confers hypersensitivity to glucose and sucrose. The *prl1* mutation results in transcriptional derepression of glucose responsive genes defining a novel suppressor function in glucose signaling. The *prl1* mutation also augments the sensitivity of plants to growth hormones including cytokinin, ethylene, abscisic acid, and auxin; stimulates the accumulation of sugars and starch in leaves; and inhibits root elongation. *PRL1* encodes a regulatory WD protein that interacts with ATHKAP2, an  $\alpha$ -importin nuclear import receptor, and is imported into the nucleus in *Arabidopsis*. Potential functional conservation of PRL1 homologs found in other eukaryotes is indicated by nuclear localization of PRL1 in monkey COS-1 cells and selective interaction of PRL1 with a nuclear protein kinase C- $\beta$ II isoenzyme involved in human insulin signaling.**

[Key Words: Glucose repression; hormone sensitivity; cell elongation; WD-40 protein;  $\alpha$ -importin]

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Coordination of signaling pathways responding to hormonal, metabolic and environmental stress stimuli has a central role in plant growth control. *Arabidopsis* seedlings developing in the dark undergo fast elongational growth until the depletion of carbon reserves of the cotyledons. For subsequent growth, seedlings require either an external carbon supply or a light signal perceived by the photoreceptors controlling photomorphogenesis and de-etiolation required for autotrophic growth (Chory et al. 1996). In particular, far-red light signaling via the photoreceptor phytochrome A is negatively regulated by sucrose via glucose repression, and this effect is alleviated by the *sun* mutations (Dijkwel et al. 1997). In addition to glucose and sucrose, photomorphogenesis is antagonized by certain plant hormones, such as brassinosteroids. In contrast, cytokinins synergistically enhance the induction of de-etiolation by light. Brassinosteroid deficiency, as well as cytokinin treatment of wild-type plants, therefore yield a phenocopy of mutations causing de-etiolation (Chory et al. 1994; Li et al. 1996; Szekeres et al. 1996). Mutations of the *COP*, *FUS*, and *DET* genes result in constitutive photomorphogenesis and de-etiolation in the dark (von Arnim and Deng 1996). *COP1* encodes a

regulatory protein carrying  $\beta$ -transducin-like WD-40 repeats. *COP1* is proposed to act as a nuclear repressor of light-regulated genes in concert with the *COP9* complex in dark-grown plants (von Arnim and Deng 1994; Chamovitz et al. 1996). Functional analogies between *COP1* and the *TUP1* WD protein, acting as a general repressor of glucose-regulated genes in yeast (Tzamarias and Struhl 1995), as well as between the *COP9* complex and the *SWI/SNF* modulators of RNA polymerase II (*Pol II*) have been noted (Chamovitz et al. 1996; Chory et al. 1996; Wilson et al. 1996). Although the role of *COP1* in glucose repression is still unknown, its cytoplasmic localization in the light suggests that *COP1* is unlikely to function as a *TUP1*-like repressor in glucose signaling of light-grown plants (von Arnim and Deng 1996).

Carbon partitioning is mediated by sucrose transport in many plant species. Growth control by carbon partitioning is therefore thought to be executed at the cellular level by glucose signaling (Stitt and Sonnewald 1995). In light-grown plants, sucrose feeding and inhibition of sucrose transport, leading to cellular sugar accumulation, result in the inhibition of photosynthesis and chlorophyll biosynthesis, defective root development, as well as induction of stress responses and accumulation of starch and anthocyanins (von Schaewen et al. 1990; Riesmeier et al. 1994; Herbers et al. 1996). As in other eu-

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karyotes, hexose phosphorylation by hexokinases is believed to provide a signal for glucose repression also in plants (Jang et al. 1997). Glucose repression down-regulates the synthesis and stability of mRNAs coding for chlorophyll a/b-binding proteins, enzymes acting in starch degradation, and Calvin and glyoxylate cycles. At the same time, glucose signaling induces the expression of genes encoding storage and defense proteins, and enzymes involved in glycolysis, nitrate assimilation, phosphate mobilization, and anthocyanin biosynthesis (Faure et al. 1994; Smeekens and Rook 1997). In cross-talk with glucose signaling, cytokinins alleviate glucose repression of the photosynthetic genes and synergistically activate the expression of glucose-induced genes. Other plant hormones may have only a secondary role in glucose responses because their synthesis is either directly or indirectly controlled by light-, glucose-, and cytokinin-signaling (for review, see Chory et al. 1996).

In addition to complex cross-talk between hormonal and metabolic regulation, genetic dissection of plant glucose signaling is confronted with the problem that plants themselves produce glucose by CO<sub>2</sub> fixation. Because light signaling is modulated by glucose and cytokinin and, vice versa, glucose and cytokinin signaling is controlled by light, mutations affecting glucose regulation may cause either lethality or severe developmental defects. Mutations relieving glucose repression are therefore expected to result in an enhanced expression of glucose responsive genes, as well as in potential defects in cytokinin signaling, root development, general stress responses, and chlorophyll and anthocyanin biosynthesis (Smeekens and Rook 1997). Here we show that such a phenotype is conferred by a recessive mutation in the *Pleiotropic regulatory locus 1 (PRL1)* encoding a conserved nuclear WD-protein that functions as a pleiotropic regulator of glucose and hormone responses in *Arabidopsis*.

## Results

### *The prl1 mutation results in altered carbon partitioning and hypersensitivity to glucose and sucrose*

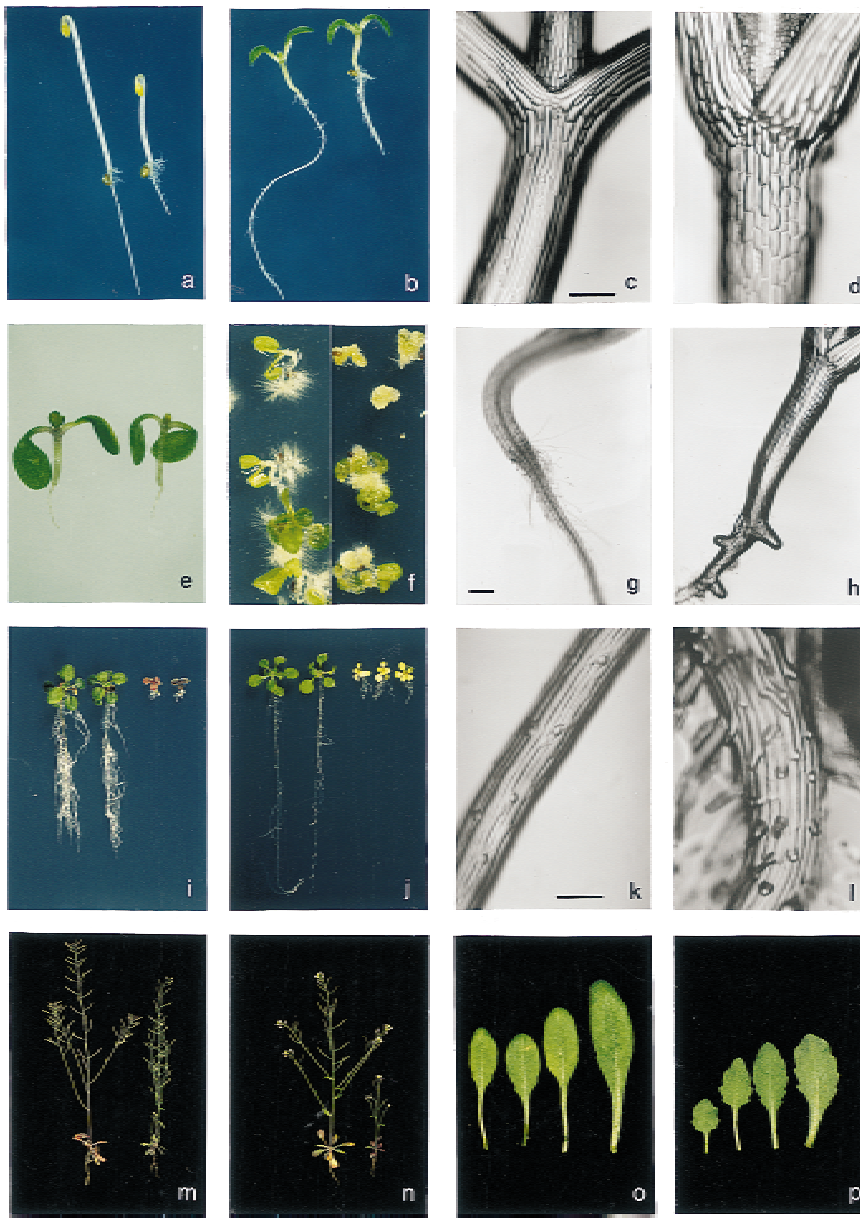
A mutant displaying growth arrest in the presence of 175 mM sucrose or glucose (Fig. 1i), but wild-type growth responses to nonmetabolizable sugars and osmolytes (listed in Materials and Methods), was identified in an *Arabidopsis* T-DNA insertional mutant collection (Koncz et al. 1992). The mutation causing glucose and sucrose hypersensitivity resulted in complex recessive phenotypic defects (Fig. 1) that cosegregated with the hygromycin resistance marker of the T-DNA-tagged locus *PRL1* mapped by genetic linkage analysis to chromosome 4-44 (see Materials and Methods). Sugar dose-growth response curves monitoring shoot and root weight, root length, and shoot/root ratio revealed no significant difference between wild-type and *prl1* plants grown in the presence of low concentrations [0.1% (3 mM) and 0.5% (15 mM)] of sucrose (Fig. 2a–d). Root elon-

gation of *prl1* was reduced two- to threefold in comparison to wild type, independent of the concentration of external carbon and nitrogen sources (Fig. 2c; data not shown). Increasing the sucrose concentration up to 4% (117 mM), however, resulted in severe inhibition of both shoot and root development. Therefore, the shoot/root ratio of *prl1* plants growing on higher than 1% sucrose did not change dramatically. On 6% (175 mM) sucrose *prl1* barely grew and lost viability within 3 weeks. In comparison with wild type, the onset of growth defects correlated with a two- to fivefold increase of free glucose, fructose, sucrose, and starch content in leaves of *prl1* seedlings grown on 2% (59 mM) and 4% sucrose (Fig. 2e–g).

### *Pleiotropic effects of the prl1 mutation on seedling development and hormonal responses*

The *prl1* mutation resulted in a two- to threefold inhibition of root elongation both in the dark and in the light (Fig. 1a,b). Hypocotyl elongation of *prl1* plants was reduced in the dark (Fig. 1a), but was comparable with that of wild-type plants in white (Fig. 1b), red, far-red, and blue light (data not shown). Hypocotyl surface imprints showed a twofold increase in number, contrasting with a twofold decrease in length, of cells in the hypocotyl epidermis of *prl1* in comparison with wild type (Fig. 1c,d). Premature initiation of side roots in light-grown *prl1* seedlings indicated an enhanced auxin sensitivity (Fig. 1g,h). In the presence of auxins, arresting the elongation of primary roots, wild-type seedlings developed numerous side-roots covered by hairs, whereas primary and adventitious roots of *prl1* were converted to undifferentiated callus tissues (Fig. 1f). In contrast with an alternating pattern of root-hair (trichoblast) and non-hair (atrachoblast) cells of wild-type root epidermis (Fig. 1k), adjacent rhizodermal cell files of *prl1* carried ectopic root hairs (Fig. 1l), a sign of augmented ethylene sensitivity (Masucci and Schiefelbein 1996). In comparison with wild type, ethylene treatment caused a fivefold reduction of hypocotyl elongation of etiolated *prl1* seedlings (Fig. 3g). When grown in soil, *prl1* seedlings clearly differed from wild type by their altered leaf morphology and serrated leaf margins (Fig. 1o,p). In contrast, in the presence of cytokinin (4.5 μM isopentenyl adenosine) and 90 mM sucrose the phenotype of light-grown *prl1* and wild-type seedlings was nearly identical (Fig. 1e). Unlike wild type plants, however, the *prl1* mutant developed short roots and accumulated 20% to 30% more chlorophyll and anthocyanin both in the presence and absence of cytokinin (data not shown).

A combination of *prl1* with the recessive *ein2* mutation and its allele *ckr1*, conferring cytokinin resistance and ethylene insensitivity (Su and Howell 1992; Ecker 1995), did not suppress the short root *prl1* phenotype. Root growth of the homozygous *prl1; ckr1* double mutant, as well as wild-type and *prl1* seedlings, was inhibited by cytokinin (2 μM 6-benzyl-aminopurine), in contrast to cytokinin resistant root elongation of the *ckr1* mutant in the light (Fig. 3h). When treated with ethylene

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**Figure 1.** Effects of the *prl1* mutation on seedling development and growth responses to glucose, cold stress, and plant hormones. In comparison with wild type (left in *a* and *b*), the *prl1* mutant (right in *a* and *b*) exhibits reduced hypocotyl elongation in the dark (*a*), and inhibition of root elongation both in the dark (*a*) and in the light (*b*). The length of barrel-shaped epidermal cells of the hypocotyl of light-grown *prl1* seedlings (*d*) is about half of that of elongated wild-type epidermal cells (*c*). When grown on cytokinin and sucrose in the light, the phenotypes of wild-type (left in *e*) and *prl1* (right in *e*) seedlings are similar. In the presence of auxin, inhibiting the elongation of primary roots, wild-type plants (left in *f*) develop side roots densely covered by hairs, whereas the roots of *prl1* seedlings (right in *f*) are converted to quickly proliferating, unorganized callus tissues. After 5 days of germination in the light, no side roots are observed on the primary root of wild-type seedlings (*g*), whereas *prl1* develops numerous adventitious root initials (*h*). In the presence of 175 mM glucose *prl1* seedlings (right in *i*) accumulate anthocyanins and loose viability in contrast to wild type (left in *i*). Unlike wild-type (left in *j*), *prl1* seedlings (right in *j*) display bleaching and growth retardation when planted in media containing 0.1  $\mu$ M ABA. In contrast with alternating files of trichoblasts and atrichoblasts on the wild-type root epidermis (*k*), adjacent rhizodermal files carry ectopic root hairs in *prl1* (*l*). At 24°C (*m*) the size of wild type (left in *m* and *n*), and *prl1* (right in *m* and *n*) is comparable, but at 14°C (*n*) *prl1* exhibits a significant growth reduction. In comparison with wild type (*o*), leaves of the *prl1* mutant (*p*) are smaller and display short petioles and serrated leaf margins. Scale bars in *c,g,k*, 200  $\mu$ m.

in the dark, the *prl1; ein2* double mutant was indistinguishable from *ein2*, displaying a long hypocotyl and an open apical hook of cotyledons in contrast to short hypocotyls and exaggerated hooks of wild-type and *prl1* (Fig. 3g). In addition to the light-dependent reversal of epistasis between *prl1* and *ein2* (*ckr1*), an unusual interaction was observed between *prl1* and the *amp1* mutation, conferring cytokinin overproduction (Chaudhury et al. 1993). The *amp1; prl1* double mutant displayed a *prl1*-like short hypocotyl and root, and *amp1*-like large, open cotyledons in the dark, indicating additivity (Fig. 3i). *amp1* severely aggravated the *prl1* phenotype in the light, however, yielding a further size reduction of root, hypocotyl, and leaf (Fig. 3j). Decreasing the temperature from 24 to 14°C also caused a growth inhibition of *prl1* (Fig. 1m,n). Cold sensitivity of *prl1* correlated with an

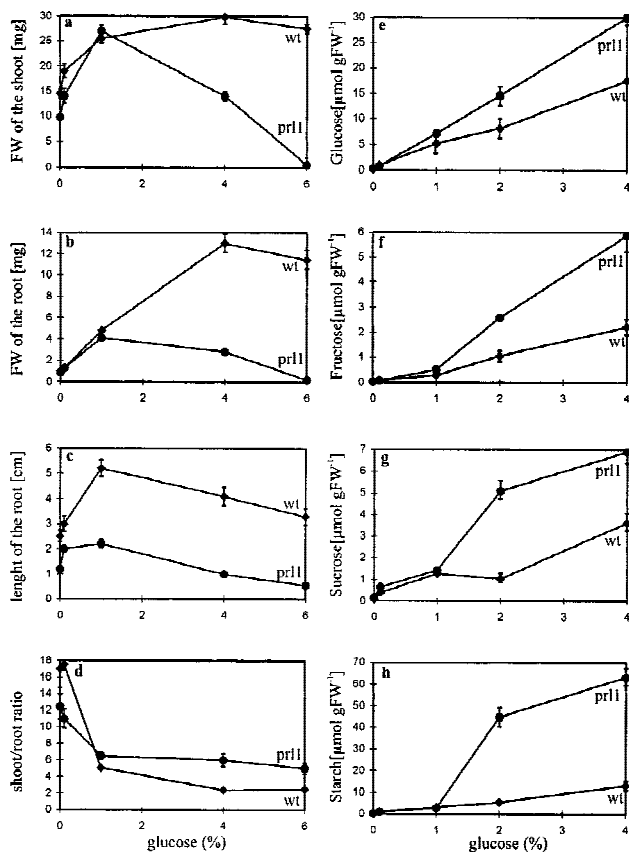
enhanced sensitivity to abscisic acid (ABA). A treatment of 5-day-old seedlings with 0.1  $\mu$ M ABA resulted in bleaching and growth reduction of *prl1* in contrast to wild type (Fig. 1j). Further assays showed that growth responses to gibberellins, brassinosteroids, methyl jasmonate, salicylic acid, phosphate, NaCl, heavy metals, heat-shock, and drought were unaffected by the *prl1* mutation (data not shown).

#### *Transcriptional derepression of genes regulated by sucrose and cytokinin in the prl1 mutant*

Northern hybridization analysis using RNAs prepared from wild-type and *prl1* plants grown in the dark or in white light (excluding UV-A and -B) on either 3 or 90 mM sucrose, with or without 4.5  $\mu$ M cytokinin, revealed a



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**Figure 2.** Sugar-dependent growth responses and carbohydrate accumulation in *prll*. Comparison of shoot (a) and root (b) weights, root lengths (c), and shoot/root ratios (d) measured in wild-type (◆) and *prll* (●) plants grown in the presence of 0, 1, 4, and 6% glucose. Accumulation of glucose (e), fructose (f), sucrose (g), and starch (h) in the leaves of wild-type (◆) and *prll* (●) plants grown in the presence of 0, 1, 2, and 4% glucose.

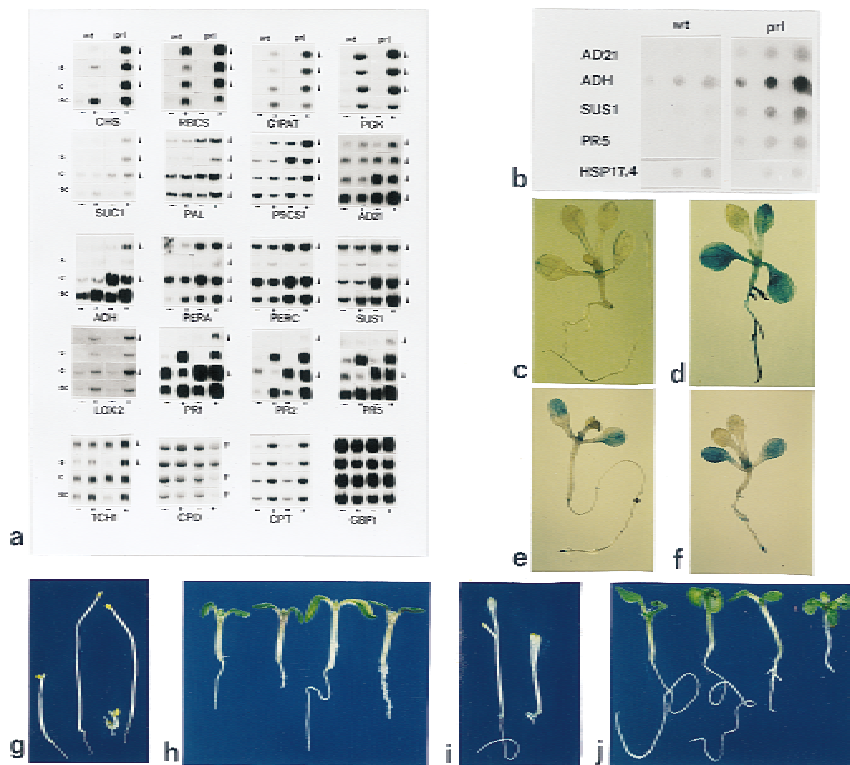
derepression of glucose- and cytokinin-regulated genes in the *prll* mutant (Fig. 3a). In accordance with an overproduction of anthocyanins, the RNA levels of chalcone synthase (*CHS*) and phenylalanine ammonia-lyase (*PAL*) genes were significantly increased in the *prll* mutant as compared with the wild type. In addition, transcript levels of the light-activated and glucose-repressed ribulose-1,5-bisphosphate carboxylase (*RBCS*), glucose-1-phosphate-adenylate transferase (*G1PAT*), and phosphoglycerate kinase (*PGK*) genes were three- to fivefold higher in *prll* than in wild-type plants grown in the absence or presence of either sucrose or cytokinin in the light. The expression of other light-regulated genes encoding, for example, chlorophyll a/b-binding proteins, glucose-6-phosphate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, glutamine/glutamate synthases, superoxide dismutases, malic enzyme,  $\text{H}^+$ /hexose transporters (data not shown), chloroplast triose-phosphate translocator (*CPT*), and bZIP transcription factors (*GBF1*, *GBF3*, *TGA1a*, and *TGA3*) showed no difference between wild-type and the *prll* mutant. In accordance with the accumulation of free sugars, one of the sucrose trans-

porter genes (*SUC1*; Sauer and Stolz 1994) was found to be active in *prll*, but not in wild-type plants grown in the absence of cytokinin. The sucrose synthase (*SUS1*), alcohol dehydrogenase (*ADH*), anionic peroxidase (*PERA*), and peroxidase C (*PERC*) genes showed derepression in the absence and enhanced induction in the presence of cytokinin in *prll*, but their activity was sucrose repressible. In contrast, the *TCH1* calmodulin gene featured a derepression on sucrose, whereas the steady-state RNA level of *LOX2* lipoxygenase was increased by cytokinin in *prll*. The abscisic acid-induced genes *AD21* and  $\Delta^1$ -pyrroline-5-carboxylate synthase 1 (*P5CS*) displayed higher expression and inducibility by glucose and cytokinin in *prll*, whereas the RNA levels of pathogenesis-related genes *PR1*, *PR2*, and *PR5* were increased by cytokinin 5- to 10-fold, but their induction by glucose and light was unaltered in *prll*. The *CPD* gene, encoding an essential enzyme in brassinosteroid biosynthesis (Szekeres et al. 1996), proved to be unique among the genes tested because its expression was down-regulated in the *prll* mutant.

Except for *AD21*, *SUS1*, *PERA*, and *PERC*, the genes affected by the *prll* mutation showed a similar steady-state mRNA level in wild type and *prll* when the seedlings were treated with both cytokinin and sucrose. To determine whether transcription or RNA stability of cytokinin and glucose regulated genes was affected by the *prll* mutation, RNA probes were synthesized in isolated nuclei prepared from wild type and *prll* plants. Hybridization of run-on RNA probes with cDNA dot-blots revealed two- to fivefold higher *PR5*, *SUS1*, *ADH*, and *AD21* transcript levels in *prll* as compared with wild type (Fig. 3b), indicating that at least part of the differences detected by Northern hybridization of steady-state RNAs was attributable to transcriptional changes caused by the *prll* mutation. To support this conclusion, a  $\beta$ -glucuronidase (*GUS*) reporter gene driven by the *ADH* promoter (Dolferus et al. 1994) was introduced into wild-type and *prll* plants. The *ADH*-*GUS* expression was confined to the meristematic junction of rosette leaves in the wild type (Fig. 3c), whereas high *ADH*-*GUS* activity was detected in leaves, vascular meristems, and roots of *prll* (Fig. 3d). The difference between *ADH*-*GUS* expression in wild-type (Fig. 3e) and *prll* (Fig. 3f) plants was alleviated by a mutation of G-box II sequences within the *ADH* promoter (Dolferus et al. 1994).

#### *prll* encodes a conserved WD protein

Southern hybridization mapping of *prll* genomic DNA with probes derived from the T-DNA tagging vector pPCV6NFluxF (Koncz et al. 1994) showed that *prll* contained a tandem repeat of three T-DNAs. Plant DNA fragments linked to the T-DNA ends (LB1 and LB3; Fig. 4a) were isolated by plasmid rescue (Koncz et al. 1990), sequenced and used as probes for the isolation of wild-type genomic and cDNA clones. Sequence comparison of genomic and cDNA clones indicated that the *PRL1* gene contained 17 exons. The transcriptional start site was located 38 bp upstream of the ATG codon as determined



**Figure 3.** Genetic interactions and effects of the *prl1* mutation on the expression of glucose and cytokinin responsive genes. (a) Northern filter quadrates were loaded with  $4 \times 4$  RNA samples of 20  $\mu\text{g}$  of each. In each quadrate, the first upper row is loaded with RNA samples prepared from wild-type (wt) and *prl1* (prl) plants grown in the presence of 0.1% (3 mM) sucrose. The second and third rows (s and c) carry RNA samples from wild-type and *prl1* plants grown in the presence of 3% (87 mM) sucrose (s), and cytokinin [*c*; 4.5  $\mu\text{M}$  isopentenyl adenosine (IPAR)], respectively. The fourth row (sc) in each quadrate contains RNA samples from wild-type and *prl1* plants subjected to combined sucrose (87 mM) and cytokinin (4.5  $\mu\text{M}$  IPAR) treatments. The first and third columns labeled by – are loaded with RNA samples from dark-grown plants, whereas the second and fourth columns of filter quadrates marked by \* carry RNAs from light-grown plants. The filters were hybridized with cDNA probes encoding chalcone synthase (CHS), ribulose-1,5-bisphosphate carboxylase (RBCS), glucose-1-phosphate-adenylate transferase (GIPAT), phosphoglycerate kinase (PGK), sucrose transporter (SUC1), phenylalanine ammonia-lyase (PAL),  $\Delta^1$ -pyrro-

line-5-carboxylate synthase (P5CS1), late-abundant embryonic protein (AD21), alcohol dehydrogenase (ADH), AP3 anionic peroxidase (PERA), peroxidase C (PERC), sucrose synthase (SUS1), lipoxygenase 2 (LOX2), pathogenesis-related proteins (PR1, PR2, and PR5), calmodulin (TCH1), C23-steroid hydroxylase (CPD), chloroplast triose-phosphate translocator (CPT), and G-box-binding factor (GBF1). (b) Dot-blot hybridization of *AD21*, *ADH*, *SUS1*, *PR5*, and *Hsp17.4* cDNAs (0.4, 2, and 4  $\mu\text{g}$  loaded in each row from left to right) with nuclear run-on RNA samples prepared from isolated wild-type (wt) and *prl1* (prl) nuclei. (c–f) Patterns of GUS expression in wild-type (c,e) and *prl1* (d,f) plants carrying a *uidA* reporter gene driven by a wild-type ADH promoter construct, CADH (c,d), and a mutant ADH promoter ( $\Delta$ G-box2; Dolferus et al. 1994) containing base-pair exchanges in the G-boxII (e,f). (g–j) Phenotypes of *prl1* double mutants. Growth response of wild-type, *ein2*, *prl1*, and *prl1*; *ein2* (double mutant) seedlings (from left to right in g) to ethylene-treatment for 5 days in the dark. Phenotype of wild-type, *prl1*, *ckr1*, and *prl1*; *ckr1* seedlings (from left to right in h) grown in the presence of cytokinin (2  $\mu\text{M}$  N<sup>6</sup>-benzylaminopurine) for 10 days in the light. Dark-grown *amp1* (left in i) and *amp1*; *prl1* (right in i) seedlings 5 days after germination. Phenotypes of wild-type, *amp1*, *prl1*, and *amp1*; *prl1* seedlings (from left to right in j) grown for 10 days in the light.

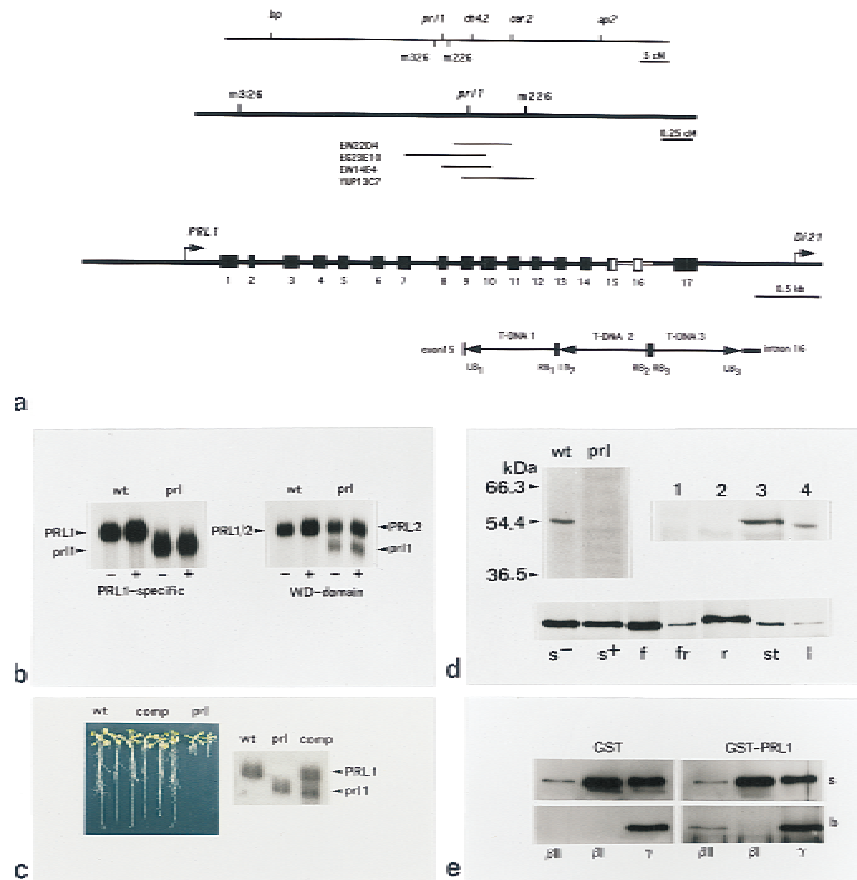
by primer extension (data not shown). Database searches revealed that the closest neighbor located 3'-downstream of *PRL1* was an ABA-induced gene, *DI21*. Sequence comparison of the wild-type and T-DNA-tagged alleles showed that the T-DNA insertion caused a deletion of sequences between exons 15 and 17, leading to a 3'-truncation of the *PRL1*-coding sequence (Fig. 4a). In addition to clones carrying the wild-type *PRL1* allele, the sequence analysis also identified genomic and cDNA clones encoding a *PRL1* homolog, *PRL2*. Alignment of *PRL1*- and *PRL2*-coding sequences, both spanning 1.65 kb, revealed four gaps of 3–12 bp upstream of codons 159 and 153, respectively. Amino-terminal segments of deduced PRL1 and PRL2 protein sequences located upstream of these positions shared only 65% identity, whereas their carboxy-terminal segments showed an amino acid identity of 89% (Fig. 5). With 5'-end-specific cDNA probes, the *PRL1* gene was found to hybridize to yeast artificial chromosome (YAC) clones EW22D4, EG23E10, EW14E4, and yUP13C7, and mapped to chro-

mosome 4-44 (Schmidt et al. 1996) confirming the results of the genetic linkage analysis. *PRL2* was mapped to YAC clones CIC4H5, CIC11H4, CIC12C2, yUP23E10, and yUP24B8 of contig *KG17* located in the vicinity of marker m560B in chromosome 3–24.

5' sequences of *PRL1* did not hybridize to the *PRL2* transcript under stringent conditions and detected only the *PRL1* mRNA of 1.75 kb in wild-type plants. The probe hybridized to a mRNA of 1.55 kb in *prl1*, providing evidence for transcription of the T-DNA-tagged mutant allele (Fig. 4b). Comparable amounts of transcripts were observed in both wild-type and *prl1* plants grown in the light in the presence or absence of 90 mM sucrose indicating that transcription of the *PRL1* and *prl1* alleles was unaffected by sucrose. In addition to the truncated *prl1* transcript, probing the same blots with 3'-cDNA sequences conserved between *PRL1* and *PRL2* detected *PRL2* mRNA of 1.75 kb in *prl1* plants indicating that transcription of *PRL2* was not affected by the *prl1* mutation.

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**Figure 4.** Characterization of wild-type and mutant alleles, genetic complementation of the *prl1* mutation, and interaction of PRL1 with human PKC- $\beta$ II in vitro. (a) The position of *prl1* on chromosome 4-44 and YAC clones located between the markers m326 and m226 are shown by the maps in the *top* two lanes. Structure of the *PRL1* gene, position of the neighboring *DI21* gene, and location of the trimeric T-DNA insertion replacing sequences between exon 15 and intron 16 in the *prl1* mutant are depicted in the *bottom* section. Left (LB) and right (RB) borders of the T-DNA units within the tandem repeat are numbered. Map distances are indicated by bars. (b) Hybridization of RNA samples prepared from wild-type (wt) and *prl1* (*prl*) plants grown in the absence (-) or presence of 3% sucrose (+) with a *PRL1*-specific probe derived from the 5' end of *PRL1* cDNA (*left* section). Hybridization of the same RNA blot with the 3'-end of *PRL1* cDNA, encoding the WD-40 repeats conserved between *PRL1* and *PRL2* (*right* section). Arrows indicate the position of *PRL1*, *PRL2* and truncated *prl1* RNAs. (c) Genetic complementation of the *prl1* mutation. Germination test (*left*) showing normal root elongation of wild-type (wt; first two seedlings) and complemented plants (comp; four seedlings to the *right* from wild type) in contrast to defective root growth of the *prl1* mutant (last two seedlings to the *right*). Northern hybridization of RNAs (*right*) prepared from wild-type (wt), *prl1* (*prl*), and complemented (comp) plants with a *PRL1*-specific probe. Positions of *PRL1* and *prl1* mRNAs are indicated by arrows. (d) Western blotting with anti-PRL1 antibody (*top left*) detects the PRL1 protein of 54.4 kDa in the wild type (wt), but shows no specific cross-reaction with proteins extracted from the *prl1* mutant. Immunoblotting of membrane proteins (*top right*) obtained from wild-type plants by extraction with (1) 50 mM NaCl, (2) 500 mM NaCl, (3) 0.2 M NaCO<sub>3</sub> (pH 11.5), and (4) 2% Triton X-100. Immunoblotting of SDS-solubilized protein extracts (*bottom*) prepared from wild-type seedlings grown in the absence (s<sup>-</sup>) or presence (s<sup>+</sup>) of 3% sucrose, flowers (f), fruits (fr), roots (r), stems (st), and leaves (l). (e) Pull-down PKC assays. PKC- $\beta$ II (1  $\mu$ g) and 3  $\mu$ g of PKC- $\beta$ I and  $\gamma$  were incubated with GST and GST-PRL1- $\Delta$ B proteins immobilized on glutathione-Sepharose. The matrices were extensively washed, then the bound protein fractions were eluted, separated by SDS-PAGE, and immunoblotted with an antibody recognizing all three PKCs. Supernatant fractions in the *top* lanes (s) show the amount of unbound PKCs. Bound fractions in the *bottom* lanes (b) indicate binding of PKC- $\beta$ II to the GST-PRL1- $\Delta$ B fusion protein, as well as an unspecific interaction of PKC- $\gamma$  with the GST bait used as internal control.



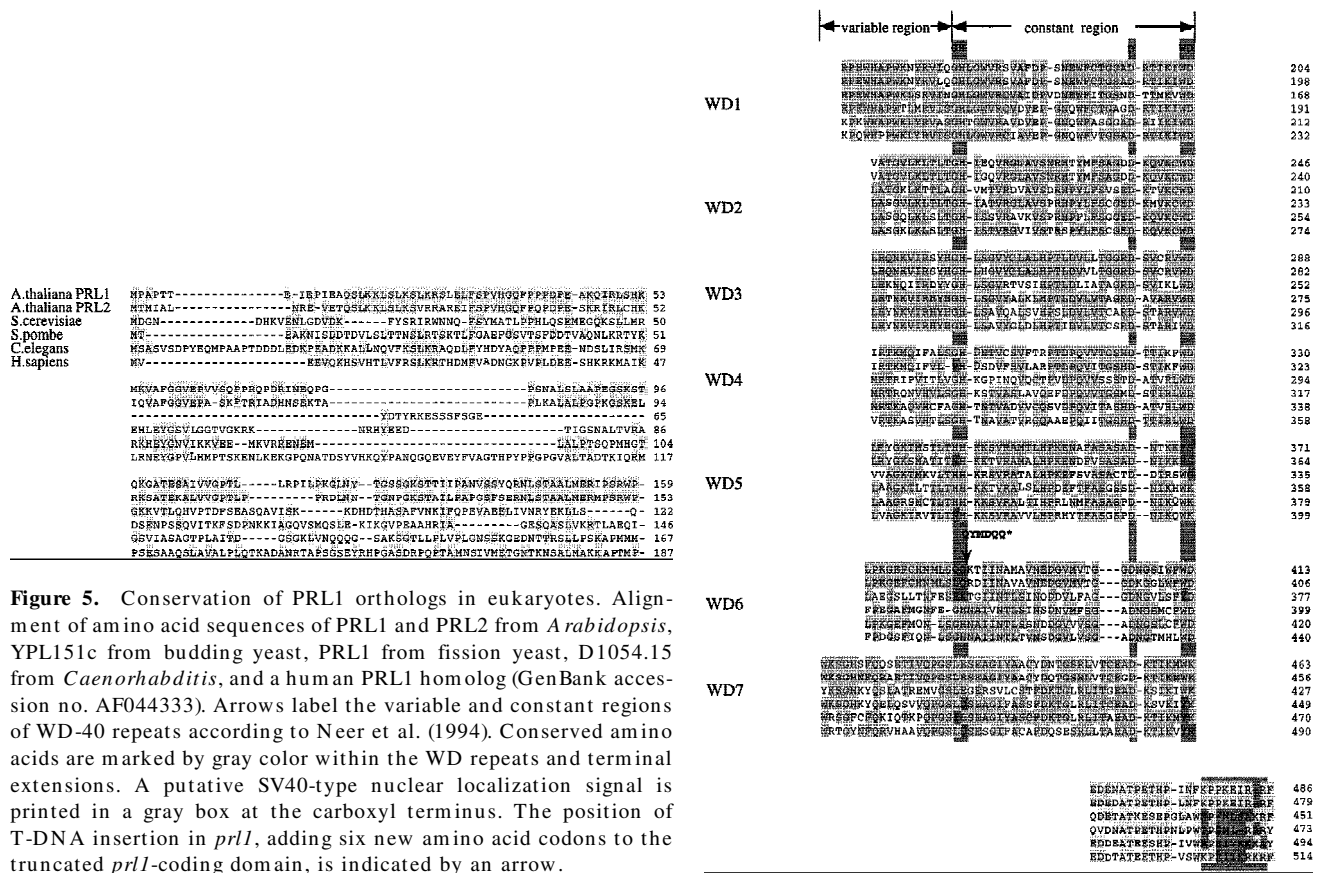
The *prl1* mutation could be complemented by transformation with the wild-type *PRL1* gene carried by the *Agrobacterium* vector pPCV002 (Koncz and Schell 1986) linked to a kanamycin resistance marker. The *prl1* mutation dramatically reduced the frequency of *Agrobacterium*-mediated transformation. Therefore, *Agrobacterium* infection of 500,000 *prl1* root explants yielded only three kanamycin-resistant transformants that could be regenerated to plants displaying wild-type phenotype concerning all visible and molecular phenotypic traits affected by the *prl1* mutation (data not shown). All three complemented lines carried a single copy of wild-type *PRL1* gene and their F<sub>2</sub> progeny showed a 3:1 segregation ratio of kanamycin resistant wild-type plants with normal root elongation and kanamycin sensitive *prl1* plants with short roots (Fig. 4c). Hybridization with the *PRL1*-

specific probe demonstrated that the complemented lines synthesized both wild-type *PRL1* and truncated *prl1* mRNAs (Fig. 4c).

Analysis of protein sequences deduced from the cDNA indicated that PRL1 is a basically charged protein of 54 kDa carrying seven carboxy-terminal  $\beta$ -transducin repeats characteristic for regulatory WD-40 repeat proteins in eukaryotes (Neer et al. 1994). In the database, PRL1 identified a family of WD proteins with unknown function: PRL2 from *Arabidopsis* shared 83%, PRL1 from fission yeast 69%, YPL151c from budding yeast 63% (Purnelle et al. 1996), and hypothetical gene product D1054.15 from *Caenorhabditis* 62% sequence identity with PRL1. Expressed sequence tags (ESTs) showing *PRL1* homology were also found in *Drosophila* and mouse. A human ortholog (GenBank accession no.



PRL1 and glucose response in *Arabidopsis*



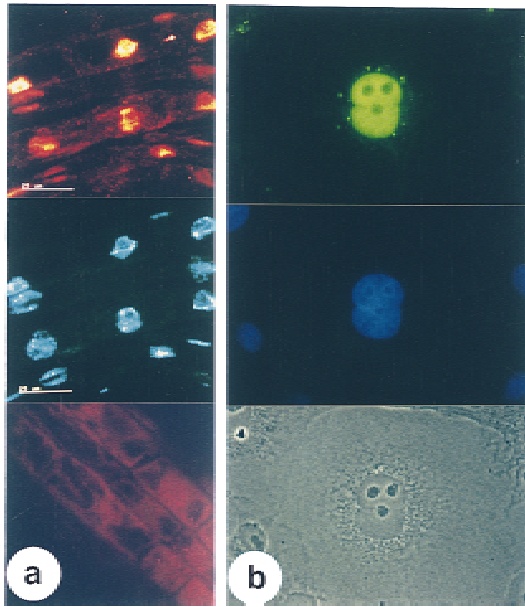
AF044333), showing 59% sequence identity with PRL1, was isolated using expressed sequence tags EST178245 and yw86d09 as probes (Fig. 5; L. Ökrész, unpubl.).

*Cellular localization of the PRL1 protein and its interaction with human PKC-βII*

Sequence analysis of the mutant *prl1* gene showed that the T-DNA insertion interrupted the *PRL1* sequence at codon position 392 (after the motif MLSQQ in the sixth WD-40 repeat; Fig. 5) and resulted in the addition of six new carboxy-terminal amino acids. An affinity-purified antibody raised against a unique PRL1 peptide (see Materials and Methods) failed to detect a truncated PRL1 protein with the predicted molecular mass of 43.4 kD in the mutant but recognized a protein of 54 kD in wild-type plants (Fig. 4d). Control experiments, using the synthetic PRL1 peptide as a competitor in immunoblotting with the anti-PRL1 antibody, confirmed that the protein of 54 kD was indeed PRL1. These experiments also demonstrated that the PRL2 protein and its fusion protein derivatives, produced in *Escherichia coli* and in yeast and lacking the PRL1-specific peptide sequence, were not recognized by the anti-PRL1 antibody (data not shown). PRL1 was detected in microsomal membrane cell fractions prepared from wild-type plants. PRL1 could only be extracted from the membranes with 0.2 M Na<sub>2</sub>CO<sub>3</sub> at pH 11.5, but not by 0.05 or 0.5 M NaCl (Fig.

4d). In SDS-solubilized extracts prepared from different organs, the total amount of PRL1 protein was found to be the highest in roots and flowers, less in stems, and the lowest in leaves (Fig. 4d). Confocal laser microscopy of immunostained *prl1* roots detected only a background signal, consistent with a lack of PRL1 protein in the mutant. In wild-type plants, some staining was associated with membrane structures, but the strongest signals overlapped with the DAPI-stained nuclei (Fig. 6a).

The remarkable conservation of PRL1 sequences in eukaryotes tempted us to construct and express a MYC-epitope-tagged PRL1 protein in green monkey COS-1 cells. Indirect immunofluorescence microscopy revealed an accumulation of PRL1 in COS-1 cell nuclei counterstained with DAPI. Some immunostaining was also associated with a filamental halo around the nuclei, whereas MYC-PRL1 was not detected in the nucleoli (Fig. 6b). Nuclear transport of PRL1 in COS-1 cells raised the question about the possible function of mammalian PRL1 orthologs. A similarity to a sequence motif mediating the interaction of RACK1 receptor with activated protein kinase C (PKC) isoenzymes in mammals (Ron et al. 1994) was found within the WD-repeats of PRL1 orthologs. Therefore, a glutathione-S-transferase fusion protein (GST-PRL1-ΔB) carrying an amino-terminal PRL1 segment of 330 amino acids was constructed, purified to homogeneity, immobilized on glutathione-Sepharose, and incubated with activated human PKC-βI,



**Figure 6.** Immunolocalization of the PRL1 protein in *Arabidopsis* and monkey COS-1 cells. (a) Confocal laser micrographs of wild-type *Arabidopsis* root cells immunostained with the anti-PRL1 antibody (top) followed by counter-staining the nuclei with DAPI (middle). Immunostaining of root cells in the *prl1* mutant (bottom). Scale bars, 25  $\mu$ m. (b) Detection of MYC-PRL1 fusion protein by indirect immunofluorescence in African green monkey COS-1 cells. Immunostaining of a COS-1 cell with anti-MYC antibody (top), counter-staining the nucleus with DAPI (middle), and light microscopic cell image (bottom).

$\beta$ II, and  $\gamma$  (Stabel et al. 1993). Following stringent washes, immunoblotting of the matrix-bound proteins revealed that GST-PRL1- $\Delta$ B retained PKC- $\beta$ II, but not PKC- $\beta$ I, whereas PKC- $\gamma$  displayed a strong binding to the control GST protein providing a suitable internal standard (Fig. 4e). Because PKC- $\beta$ I and  $\beta$ II sequences only differed by 52 carboxy-terminal amino acids (Kubo et al. 1987), the data also indicated that the carboxyl terminus of PKC- $\beta$ II was required for PRL1-binding. In addition, nuclear localization of PRL1 in COS1 cells and in vitro interaction of PRL1 with the carboxyl terminus of PKC- $\beta$ II supported the notion that the carboxyl terminus may be implicated in nuclear import of PKC- $\beta$ II in mammals (Chalfant et al. 1995; Mochly-Rosen 1995).

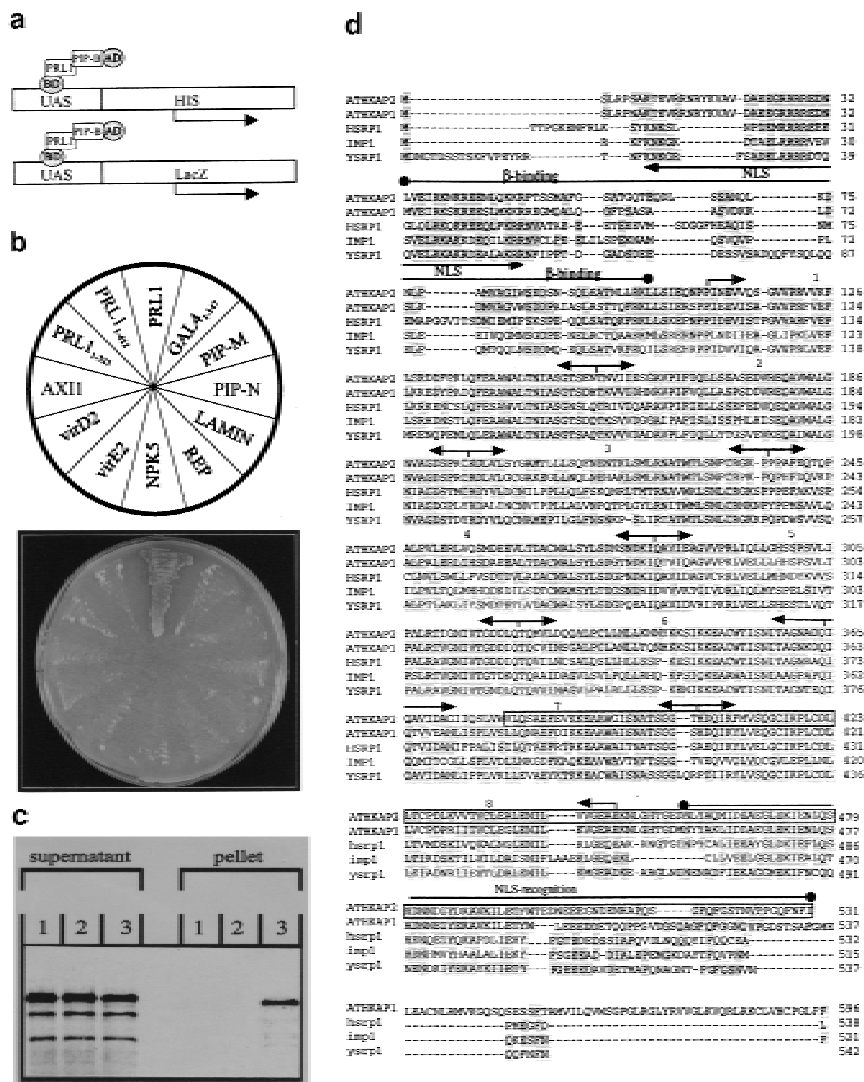
#### *PRL1* interacts with $\alpha$ -importin ATHKAP2, a novel *Arabidopsis* nuclear import receptor

To screen for *Arabidopsis* cDNAs encoding PRL1-interacting proteins (PIPs), the full-length PRL1 protein and an amino-terminal PRL1 segment of 321 amino acids were expressed as baits carrying the Gal4p DNA-binding domain in yeast using the two-hybrid vector pAS2 (Durfee et al. 1993). From  $18.4 \times 10^7$  transformants obtained with a cDNA expression library prepared from an *Arabidopsis* cell suspension in pACT2 (Durfee et al. 1993),

342 clones showed His<sup>+</sup> and LacZ<sup>+</sup> phenotype indicating an interaction between the PRL1 baits and cDNA encoded proteins fused to the activation domain in pACT2 (Fig. 7a). Classification of PIP clones identified a family of 62 cDNAs coding for carboxy-terminal segments of PIP-B corresponding to a novel class of *Arabidopsis*  $\alpha$ -importins, ATHKAP2. The amino acid sequence of ATHKAP2 deduced from a full-length cDNA of 2 kb (EMBL accession no. Y09511) showed a remarkably high sequence identity with *Arabidopsis* ATHKAP1, human HSRP1, *Xenopus* IMP1, yeast YSRP1, and other  $\alpha$ -importins involved in the nuclear import of proteins and RNAs. ATHKAP2 carried all amino-terminal sequence motifs required for nuclear localization and interaction with  $\beta$ -importins followed by eight highly conserved internal armadillo repeats (Merkle and Nagy 1997), but its carboxyl terminus was shorter than that of ATHKAP1 (Hicks et al. 1996; Fig. 7d). Using a 3'-end specific cDNA probe, the *ATHKAP2* gene was mapped to YAC clones CIC9F6 and CIC10H3 in chromosome 4-10.8. Northern analyses showed that the *ATHKAP2* mRNA levels were high in stems and flowers, but lower in leaves and roots. Similarly to *PRL1*, *ATHKAP2* mRNA levels were comparable in wild-type and *prl1* plants grown in the presence or absence of sucrose (data not shown).

The His<sup>-</sup> phenotype of yeast strains carrying an ATHKAP2-fused activation domain either alone or in fusion with unrelated proteins (NPK5, PIP-M, and PIP-N; Fig. 7b) showed that the PRL1-ATHKAP2 interaction was specific. To support these data, [<sup>35</sup>S]methionine-labeled PRL1 was synthesized by coupled transcription and translation using the cDNA template, and equal aliquots of PRL1 protein were incubated with GST-ATHKAP2 and GST proteins immobilized on glutathione-S-Sepharose, as well as with the empty Sepharose matrix (see Materials and Methods). PRL1 was quantitatively removed from the control Sepharose and GST matrices by stringent washes, but remained tightly-bound to GST-ATHKAP2, confirming an interaction of ATHKAP2 with PRL1 in vitro (Fig. 7c). A carboxy-terminal segment of ATHKAP2 of 152 amino acids interacted only with the full-length PRL1 bait in yeast, but not with truncated PRL1 proteins carrying either 321 or 412 amino-terminal amino acids. Binding of ATHKAP2 was therefore mapped to a carboxy-terminal PRL1 domain of 74 amino acids, carrying the last WD-40 repeat followed by a putative SV40-type nuclear localization signal (NLS; Figs. 5, 7b). The carboxy-terminal segment of ATHKAP2, however, interacted neither with SV40-NLS in fusion with the Gal4 DNA-binding domain nor with monopartite and bipartite NLS sequences carried by the VirD2, VirE2, lamin and AXII baits, and the replication protein (REP) of wheat-dwarf gemini virus (Fig. 7b; see Materials and Methods). The data therefore indicated that either the putative carboxy-terminal PRL1-NLS represented a specific ligand for ATHKAP2, or the interaction was not confined to recognition of PRL1-NLS by the carboxy-terminal NLS-recognition domain of ATHKAP2  $\alpha$ -importin.





**Figure 7.** Identification of  $\alpha$ -importin ATHKAP2 by screening for PIPs in the yeast two-hybrid system. (a) PRL1 in fusion with the Gal4p DNA-binding domain (BD) was used as a bait to screen for PRL1-binding proteins (e.g., PIP-B) carrying an activation domain (AD) in a yeast two-hybrid system by monitoring the activation of *His3* and *lacZ* reporter genes as described (Durfee et al. 1993). (b) A pACT2 construct, expressing a carboxy-terminal ATHKAP2 domain of 152 amino acids, was combined with different pAS2-baits encoding a full-length PRL1 protein (PRL1), amino-terminal PRL1 segments of 321 and 412 amino acids (PRL1<sub>1-321</sub> and PRL1<sub>1-412</sub>), DNA-binding domain of Gal4p in fusion with SV40 NLS (GAL4<sub>1-147</sub>), cystatin protease-inhibitor (PIP-M), unknown PRL1-binding protein (PIP-N), lamin, replicator protein of wheat-dwarf geminivirus (REP), tobacco SNF1 kinase (NPK5), tobacco AXII, and *Agrobacterium* virulence proteins VirE2 and VirD2. Specific interaction between the carboxyl terminus of ATHKAP2 and PRL1 was confirmed by growing the yeast strains on histidine-free medium. (c) A GST-fusion protein carrying the carboxy-terminal domain of ATHKAP2 and a control glutathione-S-transferase (GST) protein were immobilized on glutathione-Sepharose. Equal aliquots of the [<sup>35</sup>S]methionine PRL1 protein were incubated with the glutathione-Sepharose (1); GST (2); and GST-ATHKAP2 (3) matrices followed by extensive washing and elution of bound proteins. The labeled PRL1 protein in the supernatant and bound protein fractions was resolved by SDS-PAGE and detected by autoradiography. (d) Amino acid sequence alignment of ATHKAP2 with *Arabidopsis* ATHKAP1 (Hicks et al. 1996), human HSRP1 (GenBank accession no. U28386), *Xenopus* IMP1 (accession no. L36339), and yeast YSRP1 (accession no. M75849)  $\alpha$ -importins. Identical amino acids are labeled by gray color. Nuclear localization sequences (NLS),  $\beta$ -importin-binding, and NLS-recognition domains are marked by lines above the sequence. ARMADILLO repeats are numbered and labeled by arrow-headed separating lines. The carboxy-terminal domain of ATHKAP2 used in the yeast two-hybrid tests is boxed.

*bidopsis* ATHKAP1 (Hicks et al. 1996), human HSRP1 (GenBank accession no. U28386), *Xenopus* IMP1 (accession no. L36339), and yeast YSRP1 (accession no. M75849)  $\alpha$ -importins. Identical amino acids are labeled by gray color. Nuclear localization sequences (NLS),  $\beta$ -importin-binding, and NLS-recognition domains are marked by lines above the sequence. ARMADILLO repeats are numbered and labeled by arrow-headed separating lines. The carboxy-terminal domain of ATHKAP2 used in the yeast two-hybrid tests is boxed.

**Discussion**

*Implication of PRL1 in glucose regulation*

Glucose repression has a major role in the regulation of carbon metabolism in higher plants as in other organisms (Sheen 1990; Stitt and Sonnewald 1995). Feeding of plants with glucose or sucrose (which is converted to glucose and fructose) results in either transcriptional or post-transcriptional down-regulation (or both) of genes involved in chlorophyll biosynthesis, Calvin cycle, gluconeogenesis, starch degradation and glyoxylate cycle, but leads to the activation of genes in glycolysis, defense responses, nitrate and phosphate metabolism, and biosynthesis of anthocyanin pigments and storage proteins (for review, see Koch 1996; Jang and Sheen 1997). *Arabi-*

*dopsis* plants can tolerate as high as 300–400 mM glucose or sucrose present in growth media (Smeekens and Rook 1997). Therefore, mutations affecting potential regulatory functions in glucose signaling can simply be isolated by screening for plants showing hypersensitive or insensitive growth response to glucose or sucrose. Using this strategy, we have isolated a mutation, *prl1*, from a T-DNA-tagged *Arabidopsis* collection (Koncz et al. 1992) that displays a hypersensitive growth arrest and ultimate lethality in the presence of 175 mM glucose or sucrose. Remarkable accumulation of glucose, fructose, starch, chlorophyll, and anthocyanin pigments in the leaves suggested that the *prl1* mutation may relieve glucose repression of metabolic pathways and simultaneously enhance the activation of other pathways by glu-

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ucose. Therefore, we tested the steady-state transcript levels of numerous genes in the *prl1* mutant that were reported to be either repressed or induced by glucose in plants. It was found that many genes that are repressed or induced by glucose showed higher steady-state mRNA levels in *prl1* in comparison with the wild type. For example, among the glucose-repressible genes, the light-induced ribulose 1,5-bisphosphate carboxylase, glucose-1-phosphate-adenylate transferase, and phosphoglycerate kinase genes acting in photosynthesis, the light- and cytokinin-inducible *PAL1* gene required for flavonoid biosynthesis, the cytokinin-inducible alcohol dehydrogenase, sucrose synthase and peroxidase (*PERA* and *PERC*) genes, the *LOX2* gene involved in jasmonate synthesis, the abscisic acid and salt-regulated *P5CS1* gene controlling proline biosynthesis, the *TCH1* calmodulin gene implicated in  $\text{Ca}^{2+}$  signaling and the sucrose transporter *SUC1* gene showed de-repressed expression in the *prl1* mutant. Similarly, several glucose-inducible genes, such as the chalcone synthase gene in anthocyanin biosynthesis, the *AD21* gene coding for an embryo-specific late-abundant protein, and the cytokinin-inducible pathogenesis-related *PR* genes displayed higher transcript levels in the *prl1* mutant as compared with the wild type. These data suggested that *PRL1* may act as a negative regulator of glucose responsive genes. To support this conclusion, a hybridization analysis with nuclear run-on RNAs was performed that confirmed at least for four different genes (*ADH*, *AD21*, *SUS1*, and *PR5*) that derepression of gene expression was indeed attributable to transcriptional changes caused by the *prl1* mutation. Furthermore, a mutation in the G-boxII bZIP-binding site within the *ADH* promoter (Schindler et al. 1992; Dolferus et al. 1994) was demonstrated to alleviate the differences observed in *ADH* gene expression between *prl1* and wild-type plants by defining a common promoter upstream element required for negative regulation of transcription by *PRL1*.

So far, most known regulatory functions required for glucose repression have been identified by genetic dissection of glucose signaling in yeast and molecular studies of glucose-controlled insulin production in pancreatic  $\beta$ -cells. In addition to important functions of different hexose transporters, hexokinases (*HXK2* in yeast and glucokinase in pancreas) were found to be essential for monitoring the rate of hexose phosphorylation and thus generating a signal for glucose repression. As in yeast and pancreatic  $\beta$ -cells, inhibition of the hexokinase was demonstrated recently to relieve glucose repression causing glucose insensitivity, whereas overexpression of hexokinase was found to augment glucose repression resulting in glucose hypersensitivity in *Arabidopsis* (Jang et al. 1997). In addition to *HXK2*, many other signaling functions, such as *GRR1*, *RTG1*, *GLC7*, *REG1*, *MIG1*, *TUPI*, and *SSN6*, were demonstrated to mediate glucose repression in yeast (Johnston and Carlson 1992; Özcan and Johnston 1995; Ronne 1995). The deficiency of these functions in yeast leads to derepression of glucose responsive genes resulting in glucose insensitivity. Therefore, *PRL1* clearly differs from these regulators of glucose

repression, because the *prl1* mutation causes glucose hypersensitivity by simultaneous derepression of glucose-regulated genes.

To explain how a repressor mutation, such as *prl1*, can increase glucose sensitivity, we follow a model suggesting that *PRL1* acts as a negative regulator of a function that counteracts the activity of factors that mediate glucose repression. The serine/threonine kinase SNF1 and its activator subunit SNF4 are known to perform such a function in yeast. By controlling its phosphorylation and nuclear import, SNF1 inactivates MIG1, which acts as a negative regulator of glucose responsive genes by binding to their promoters and recruiting the TUP1/SSN6 general repressors (Trietel and Carlson 1995). The fact that a similar regulatory mechanism exists in plants is indicated by the involvement of SNF1-like kinases in the control of key metabolic enzymes (Huber et al. 1994), as well as by the characterization of plant protein kinases that can functionally complement the *snf1* mutation and interact with several regulatory subunits of SNF1 in yeast (Jiang and Carlson 1997).

#### *Pleiotropic effects of the prl1 mutation*

Characterization of the *prl1* mutation indicated that tight cross talk exists between glucose, cytokinin, and light signaling. Thus, simultaneous cytokinin and sucrose treatment of wild-type plants resulted in a *prl1*-like mutant phenocopy and abolished the differences seen in gene expression between wild-type and *prl1* plants. Furthermore, most developmental, hormonal and molecular alterations caused by the *prl1* mutation were detectable only in light-grown plants, suggesting a possible light-dependence of the *PRL1* regulatory function. Nonetheless, *PRL1* probably acts downstream and independently of the photoreceptor-mediated light signaling pathways because the *prl1* mutant shows normal light responses in hypocotyl elongation assays, and because mutations causing constitutive photomorphogenesis and de-etiolation, including *cop1* and *det1*, are epistatic to *prl1* (C. Koncz, unpubl.).

Because cytokinin is known to counteract, rather than enhance, glucose repression of the light-regulated genes, it is unlikely that *PRL1* acts as a cytokinin-dependent repressor. It is more likely that cytokinin signaling converges on a function that can alleviate glucose repression, and that *PRL1* is a light-dependent negative regulator of this function. Genetic data derived from preliminary analyses of *prl1* double mutants seem to support this model because in light-grown plants *prl1* is epistatic to the *ein2* (*ckr1*) mutation that confers cytokinin resistance, whereas the *amp1* mutation, which activates cytokinin signaling by stimulation of the synthesis of this hormone (Chaudhury et al. 1993), severely aggravates the phenotype of the *prl1* mutant. A light dependence of these genetic interactions is indicated by the observations that the *prl1*; *ein2* double mutant shows *ein2* phenotype in the dark, but *prl1* phenotype in the light, whereas the *amp1*; *prl1* double mutant displays an additivity of phenotypic traits in the dark in contrast to the

'super-*prl1*' phenotype in the light. Cross talk mediated by *EIN2* between ethylene and cytokinin signaling (Ecker 1995) may therefore provide a possible explanation for the ethylene hypersensitive phenotype and ectopic root hair development caused by the *prl1* mutation. The fact that *prl1* also augments the sensitivity of plants to ABA and auxin indicates cross talk with other hormonal signaling pathways. Auxin-mediated induction of lateral root development observed in wild-type *Arabidopsis* plants thus occurs in the absence of auxin stimulus in the *prl1* mutant correlating with its increased auxin-sensitivity. Down-regulation of the *CPD* gene encoding a steroid C23-hydroxylase suggests that *PRL1* may even modulate (in this case positively) the biosynthesis of brassinosteroids that have an essential role in skotomorphogenic development and antagonize de-etiolation (Szekeres et al. 1996). The observation that *prl1* affects the transcription of genes such as *CHS* and *PAL* implicated in UV light and fungal elicitor-induced signaling, *TCH1* in touch-signaling, *PR* genes in salicylic acid signaling, *ADH* in cold-stress signaling, and *P5CS1* and *AD21* in abscisic acid and salt signaling (for review, see Meyerowitz and Somerville 1994) also illustrates a role of *PRL1* in modulating genes controlled by other regulatory pathways. Sensitivity of the *prl1* mutant to low-temperature stress therefore not only correlates with the ABA hypersensitive phenotype, but also suggests that *PRL1* may pleiotropically affect the regulation of cell elongation. In fact, the *prl1* mutation results in the inhibition of root elongation, a phenotype that is not glucose-dependent and cannot be compensated by known plant hormones. It is not surprising that mutations in glucose signaling affect the regulation of cell shape and elongation pleiotropically, as *GRR1* and *REG1* in yeast are known to control cell size and polarity independently of their function in glucose repression (Ronne 1995). A possible conservation of *PRL1*-like functions is not only indicated by the identification of *PRL1* orthologs in yeast, but also by the recent observations demonstrating that overexpression of truncated *PRL1* proteins or antisense transcripts in fission yeast result in large, barrel-shape budding cells displaying a loss of polarity (Xia et al. 1996; unpubl.).

#### Nuclear transport and interacting partners of *PRL1*

*PRL1* encodes a novel protein carrying seven WD-40 repeats that share homology with the  $\beta$ -subunits of trimeric GTP-binding proteins and many other WD-proteins that perform different regulatory functions in eukaryotes (Neer et al. 1994). WD-40 repeats and terminal extensions of *PRL1* are distinct from those of so far characterized WD-proteins indicating that *PRL1* represents a novel class of regulatory factors. Nonetheless, connections between *PRL1* and regulation of transcription suggest some functional analogies with the *Arabidopsis* *COP1* and yeast *TUP1* WD proteins. *TUP1*, together with *SSN6*, forms a repressor complex with the *MIG1* transcription factor (Trietel and Carlson 1995; Tzamaris and Struhl 1995), whereas *COP1*, a repressor of

photomorphogenic development, directly interacts with and negatively regulates the function of the *HY5* bZIP transcription factor that binds to G-box sequences within the *Arabidopsis* chalcone synthase promoter (Ang et al. 1998). Because transcriptional regulation by *PRL1* also converges on a G-box sequence within the *ADH* promoter (Dolferus et al. 1994), it would be interesting to determine whether *PRL1* can interact with bZIP-like transcription factors. In particular, possible interaction of *PRL1* with *HY5* needs to be tested because both *prl1* and *hy5* mutations induce the initiation of lateral roots in *Arabidopsis*.

As expected for a regulatory protein functioning as a potential repressor, *PRL1* is a basically charged protein that is imported into the nucleus, but also detected in association with membrane fractions consisting of fragments of nuclear envelope and endoplasmic reticulum. *PRL1* interacts with *ATHKAP2*, a novel class of *Arabidopsis*  $\alpha$ -importins in the yeast two-hybrid system and in vitro. Although *ATHKAP2* shares >90% sequence identity with other eukaryotic  $\alpha$ -importins, it cannot recognize proteins carrying prototypes of monopartite and bipartite nuclear localization signals in the yeast two-hybrid system. Albeit *PRL1* contains a putative SV40-type NLS within its carboxyl terminus, the binding of *PRL1* to *ATHKAP2* probably reflects a more intricate, possibly regulatory, interaction suggesting a potential role for *PRL1* in regulation of nuclear import. *PRL1* orthologs, sharing >55% sequence identity are not only found in budding and fission yeasts, but also in *Caenorhabditis*, *Drosophila*, mouse, and man. Therefore, it may be relevant that *PRL1* serves as heterologous receptor in vitro for a nuclear protein kinase C- $\beta$ II isoenzyme. In addition to nuclear import of *PRL1* in COS-1 cells, selective interaction of *PRL1* with human PKC- $\beta$ II, but not with PKC- $\beta$ I, is intriguing because PKC- $\beta$ I and  $\beta$ II differ only by 52 carboxy-terminal amino acids (Kubo et al. 1987) required for nuclear import of PKC- $\beta$ II during insulin signaling (Chalfant et al. 1995; Mochly-Rosen 1995). Carboxy-terminal sequences of *Arabidopsis* *PRL1* bind a highly conserved  $\alpha$ -importin nuclear receptor, *ATHKAP2*, and share a high sequence identity with a human *PRL1* ortholog. Therefore, binding of the carboxyl terminus of PKC- $\beta$ II to the amino-terminal domain of *PRL1* in a complex with  $\alpha$ -importin may mediate nuclear targeting of PKC- $\beta$ II. Although protein interactions in heterologous systems have to be interpreted with caution, further functional study of *PRL1*-homologs in eukaryotes certainly deserves attention.

#### Materials and methods

##### *Mutant selection, physiological assays, genetic analysis, and physical mapping*

Seeds from 1200 M2 families of T-DNA-tagged *Arabidopsis* lines were germinated in MS medium (Koncz et al. 1994) containing either glucose or sucrose (0.1, 0.5, 2, 4, 6, 8, or 10%). Mutants showing growth retardation on glucose and sucrose were further tested by germination in the presence of fructose, raffinose, mannose, galactose, lactose, maltose, xylose, ribose,



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mannitol, sorbitol (each used at concentrations 1, 5, 10, 50, 100 and 200 mM), 3-*O*-methylglucose (0.1, 1, 5, 10, 50, 100 mM), 6-deoxyglucose (0.001, 0.01, 0.05 mM), and polyethylene-glycol [0.1, 0.5, 1, and 2% (weight/volume)]. Other growth responses were assayed by supplementing the media with auxins (2,4-dichlorophenoxyacetic acid or 1-naphthaleneacetic acid), cytokinins [ $N^6$ -(2-isopentenyl)adenosine riboside or  $N^6$ -benzyladenine], ABA, salicylic acid, methyl-jasmonate, brassinosteroids (each used at concentrations 0.01, 0.1, 0.5, 1.0, 2.0, and 4.5  $\mu$ M), gibberellins (0.5, 1.0, or 5.0  $\mu$ M GA3, GA4, or GA7), ethephone (25 or 50 mg/l), NaCl, KCl, KNO<sub>3</sub>, K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub>, LiCl, and CsCl (50 to 500 mM). Physiological parameters, including shoot and root weight, root length, and sugar and starch content were determined by growing plants in either MS or Hoagland media containing different concentrations of glucose at 24°C under 200 to 400  $\mu$ Einstein m<sup>-2</sup>s<sup>-1</sup> irradiance for 2, 3, or 4 weeks using 16-hr light and 8-hr dark cycle as described (Krapp et al. 1993). Data in dose response curves indicate the mean  $\pm$  standard deviation of four independent metabolite assays or six to 16 biomass measurements. Chlorophyll and anthocyanin concentrations were determined according to Chory (1992). Histological analysis and preparation of contact surface imprints were as described (Szekeres et al. 1996).

Cosegregation analysis of phenotypic traits and T-DNA-encoded antibiotic resistance markers was performed as described (Koncz et al. 1990). Seeds from 199 F<sub>2</sub> families (18464) obtained by crossing of *prl1* with wild type yielded 4603 hygromycin sensitive and 9239 hygromycin-resistant wild type, and 4622 hygromycin resistant *prl1* segregants (1:2:1 ratio,  $P = 0.999$ ). A cross between *ch42(a)* and *prl1(b)* in repulsion resulted in 4 AABb, 163 AaBB, 1553 aaBB, 165 AABb, 3008 AaBb, 165 aaBb, 1514 AaBb, 161 Aabb, and 5 aabb F<sub>3</sub> progeny, resulting in a map distance  $D = 5.13 \pm 0.2$  cM ( $P = 0.985$ ,  $\chi^2 = 1.443$ , d.f. 7). A four-point test cross between a *bp1/prl1/cer2/ap2* line and F<sub>1</sub> yielded the following parental (P) and recombinant (R) classes within the intervals: *bp/prl1* ( $P = 1304$ ,  $R = 536$ ), *prl1/cer2* ( $P = 1651$ ,  $R = 189$ ), *cer2/ap2* ( $P = 1537$ ,  $R = 303$ ), *prl1/ap2* ( $P = 1380$ ,  $R = 460$ ), *bp1/cer2* ( $P = 1167$ ,  $R = 673$ ), and *bp1/ap2* ( $P = 1068$ ,  $R = 772$ ). Classes of double crossover were between *bp1-prl1-ap2* ( $P = 1820$ ;  $R = 20$ ), *prl1-cer2-ap2* ( $P = 1825$ ,  $R = 15$ ), and *bp1-prl1-cer2-ap2* ( $P = 1752$ ,  $R = 88$ ), whereas a triple crossover class was *bp1-prl1-cer2-ap2* ( $P = 1838$ ,  $R = 2$ ). Calculation of recombination frequencies and derived map distances, as well as determination of physical map positions of *PRL1*, *PRL2*, and *ATHKAP2* genes using colony-filter hybridization of YAC libraries with cDNA probes were as described (Szekeres et al. 1996). Double mutants were constructed by crossing *prl1* with *ein2 (ckr1)*; Ecker 1995) and *amp1* (Chaudhury et al. 1993), followed by isolation of homozygous *ein2* and *amp1* lines carrying the hygromycin resistance marker of the *prl1* locus. The *prl1* double mutants were germinated in either dark or light in seed medium or in media containing either cytokinin (4.5  $\mu$ M  $N^6$ -benzyladenine) or ethephone (25 mg/l) as described (Su and Howell 1992; Szekeres et al. 1996).

#### Characterization of PRL1 alleles and genetic complementation of the *prl1* mutation

The *prl1* locus was mapped by Southern hybridization using fragments of the T-DNA vector pPCV6NFLuxF as probes (Koncz et al. 1994). Plant DNA fragments flanking the T-DNA ends were isolated and used as probes for isolation of genomic and cDNA clones from *Arabidopsis*  $\lambda$ EMBL3 and  $\lambda$ gt10 libraries, respectively, as described (Koncz et al. 1990). The screening resulted in 16 cDNA clones that all but one showed perfect sequence identity with plant DNA segments of the T-DNA

tagged *prl1* locus. The longest *PRL1* cDNA of 1742 bp (EMBL accession no. X82825) carried 21 bp corresponding to the 5'-untranslated leader of mRNA. One cDNA clone carried a full-length coding sequence of *PRL2* (EMBL accession no. X82826). Genomic clones (85) hybridizing with the *PRL1* and *PRL2* cDNAs were isolated and fingerprinted. *PRL1* genomic clones (6) were subjected to physical mapping followed by sequencing of overlapping DNA fragments covering a region of  $\sim$ 10 kb, including the *PRL1* gene of 5455 bp (EMBL accession no. X82824) and a neighboring gene, *DI21* (EMBL accession no. Z97339). Sequence comparison of wild-type and mutant loci showed that the T-DNA insertion in *prl1* deleted a segment of 344 bp from the *PRL1* gene. Breakpoints of this deletion (marked by the T-DNA ends LB1 and LB3; Fig. 4a) were located, respectively, 10 bp 3'-downstream from the 5'-end of exon 15 and 96 bp downstream from the 3' end of exon 16 of *PRL1*. *PRL1* homologs were identified in the database using the BLASTN and BLASTX programs. A cDNA encoding a human PRL1 ortholog was isolated using the ESTs EST178245 and yw86d09 as probes and sequenced (GenBank accession no. AF044333).

To complement the *prl1* mutation genetically, a *SpeI-XbaI* fragment of 7.9 kb spanning the entire *PRL1* gene was isolated from the genomic clone ppcPRL16, cloned into the *XbaI* site of pPCV002 (Koncz and Schell 1986), transferred to *Agrobacterium* GV3101 (pMP90RK), and used for transformation of root explants of the homozygous hygromycin-resistant *prl1* mutant as described (Koncz et al. 1994). The copy number of pPCV002-*PRL1* T-DNA construct was determined as described (Koncz et al. 1990). All three complemented lines carried a single copy of wild-type *PRL1* gene in linkage with a selectable kanamycin resistance marker of pPCV002 T-DNA. By selfing of these lines, 36 F<sub>2</sub> families were obtained that showed a 3:1 segregation of 2354 kanamycin-resistant wild-type and 793 kanamycin-sensitive *prl1* F<sub>3</sub> progeny.

#### Analysis of gene expression in the *prl1* mutant and immunolocalization of the PRL1 protein

RNA isolation from *Arabidopsis* and Northern filter hybridizations with CHS (GenBank accession no. M20308), *RBCS* (accession no. X13611), *GIPAT* (accession no. T46127), *PGK* (accession no. T04348), *SUC1* (Sauer and Stolz 1994), *PAL* (accession no. L33677), *P5CS1* (accession no. X86778), *AD21* (AtDi21; EMBL accession no. X78585), *ADH* (GenBank accession no. M12196), *PERA* (accession no. M58380), *PERC* (accession no. T03969), *SUS1* (Martin et al. 1993), *LOX2* (Bell and Mullett 1993), *PR1*, *PR2*, *PR5* (Uknes et al. 1992), *TCH1* (Braam 1992), *CPD* (Szekeres et al. 1996), *CPT* (accession no. T04248), *GBF1* (Schindler et al. 1992) and other cDNA probes were as described (Szekeres et al. 1996). Nuclei were isolated from wild-type and *prl1* plants and run-on transcripts were labeled with <sup>32</sup>P[UTP] in vitro (Somssich 1994). Nuclear run-on RNA probes, showing equal specific activity, were hybridized to dot-blots loaded with aliquots (0.4, 2, and 4  $\mu$ g) of *PR5*, *SUS1*, *ADH*, *AD21*, and *Hsp17.4* (EMBL accession no. X17293) cDNAs (Fig. 3b). The activity of *ADH* promoter-driven GUS reporter constructs (CADH and  $\Delta$  G-box-2; Dolferus et al. 1994) was assayed by histochemical staining of *Arabidopsis* seedlings for 6 hr with X-gluc [1 mg/liter in 50 mM Na-phosphate buffer (pH 7.0) containing 0.5 mM K-ferricyanide and 0.5 mM K-ferrocyanide] as described (Mathur et al. 1998).

From a polyclonal serum raised against a PRL1-specific peptide (VVSQPPRQPDRINEQPGPS located between amino acid positions 64 and 83; Fig. 5) in rabbit, an IgG fraction was purified by (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>-fractionation, protein A-Sepharose binding, and affinity chromatography on the PRL1 peptide (15 mg) coupled to

Affigel 10 (Bio-Rad) as described (Harlow and Lane 1988). A *Bam*HI fragment of *PRL1* cDNA, carrying 5'-coding sequences of 1 kb, was cloned in pGEX-2T (Pharmacia) and transformed into *E. coli* BL21DE3 (Novagen, UK) to purify a GST-PRL1- $\Delta$ B fusion protein on glutathione-S-Sepharose (Pharmacia; Ausubel et al. 1989). Peptide competition assays were performed with immunoblotted GST-PRL1- $\Delta$ B protein using anti-PRL1 IgG incubated with various amounts of PRL1 peptide, as well as with control peptides from PRL2 and other unrelated proteins. SDS-solubilized protein extracts were prepared from plant organs using a glass homogenizer and extraction buffer [100 mM Tris-HCl (pH 6.8) 0.6 M dithiothreitol, 1% SDS, 0.01% bromophenol blue], boiled for 10 min, pelleted by centrifugation (20,000g for 15 min) and separated by SDS-PAGE after loading 30  $\mu$ g protein in each lane (Ausubel et al. 1989). Crude membrane fractions were prepared from *Arabidopsis* cells maintained in a suspension culture by disruption of cells in extraction buffer [20 mM Tris-HCl (pH 8.0), 50 mM NaCl, 2 mM EDTA, and 1 mM PMSF], and separation of soluble and membrane fractions by centrifugation (100,000g, for 1 hr at 2°C). The membrane fraction was extracted with 500 mM NaCl (at 0°C for 1 hr), pelleted, and re-extracted with 0.2 M Na<sub>2</sub>CO<sub>3</sub> (pH 11.5). The final membrane pellet was solubilized with extraction buffer containing 2% Triton X-100, and together with the extracted protein fractions subjected to SDS-PAGE separation followed by immunoblotting with anti-PRL1 IgG. Microsomal and plasma membranes were purified as described (Larsson et al. 1987).

For immunolocalization, 4-day-old wild-type and *prl1* seedlings were fixed in MTBS [50 mM PIPES (pH 7.0), 5 mM EGTA, 5 mM MgSO<sub>4</sub>] containing 4% paraformaldehyde for 1 hr, washed with 0.5 ml MTBS, incubated with 2% Driselase in MTBS for 15 min to digest the cell walls, treated with MTBS containing 10% DMSO and 3% NP-40 to permeabilize the membranes, and washed four times with MTBS. The specimens were incubated for 1 hr with a 1:300 dilution of anti-PRL1 IgG, then treated for 1 hr with an anti-rabbit Cy3 antibody (1:300 dilution) in the dark, washed four times with MTBS, stained with DAPI (1  $\mu$ g/ml in H<sub>2</sub>O), and inspected by confocal laser microscopy.

The PRL1 cDNA was cloned into the *Bam*HI site of the MYC-tag expression vector pEFmPLINK (Marais et al. 1995), then 15  $\mu$ g of DNA was transfected by Lipofectin (Life Technologies, Grand Island, NY) into 2  $\times$  10<sup>5</sup> COS-1 cells on cover slips. After 48 hr, the cells were fixed in 3.5% paraformaldehyde in PBS, and permeabilized with 0.1% Triton X-100 in PBS for 20 min. The specimens were incubated with anti-MYC antibody 9E10 (Evan et al. 1985), followed by treatment with a fluorescein-labeled anti-mouse IgG (Amersham) and staining of the nuclei with DAPI (Puls et al. 1997).

#### *Analysis of PRL1 protein interactions in vitro and in the yeast two-hybrid system*

Human PKC- $\beta$ I and  $\beta$ II enzymes were expressed and purified as described (Stabel et al. 1993). Purified GST and GST-PRL1 $\Delta$ B proteins were bound to glutathione-S-Sepharose and incubated with PKC- $\beta$ I (3  $\mu$ g) and  $\beta$ II (1  $\mu$ g) activated with phosphatidylserine and phorbol-12-myristate-13-acetate in binding buffer [20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM CaCl<sub>2</sub>, 0.1% Triton X-100, and 1 mg/ml of bovine serum albumin] for 1 hr at 4°C as described (Puls et al. 1997). The beads were washed extensively with TENNS [2.5 mM Tris-HCl (pH 7.4), 2.5 mM EDTA, 250 mM NaCl, 1% NP-40, and 2.5% sucrose], then the bound proteins were eluted with 6 $\times$  SDS-sample buffer, resolved by SDS-PAGE (Ausubel et al. 1989) and immunoblotted using an anti-PKC- $\beta$  antibody (Stabel et al. 1993) and ECL detection kit (Amersham).

The *PRL1*-coding sequence was PCR amplified as a *Bam*HI-*Xho*I fragment, sequenced, and cloned into the yeast vector pAS2 by generating a fusion between PRL1 and the DNA-binding (DB) domain of Gal4p (Durfee et al. 1993). The pAS2-PRL1 bait was transformed into the yeast strain Y190, and the Gal4p-DB-PRL1 expression was confirmed by immunoblotting with an anti-Gal4p-DB antibody (Clontech). An oligo(dT) primed cDNA library was prepared in plasmid pACT2 using mRNA from an *Arabidopsis* cell suspension and a cDNA synthesis kit (BRL). Yeast host Y190 carrying the pAS2-PRL1 bait was transformed with 0.3 mg of DNA from the pACT2 cDNA library, then the cells were plated on SD-medium containing 50 mM 3-aminotriazole (3-AT) and lacking leucine, tryptophan, and histidine (Durfee et al. 1993). Transformants were inoculated on nylon filters and grown on SD-plates with 25 mM 3-AT to verify their LacZ<sup>+</sup> phenotype by  $\beta$ -galactosidase assays. pACT clones coding for PIPs were isolated and transformed into yeast strains Y187 and Y190 (Durfee et al. 1993) carrying either no bait, pAS2-PRL1, or different control baits coding for amino-terminal PRL1 segments of 321 and 412 amino acids, replicator protein of wheat dwarf geminivirus (GenBank accession no. S49387), lamin (Matchmaker System, Clontech), VirD2 (accession no. P18592), VirE2 (accession no. S11844), NPK5 (accession no. D26602), AXII (accession no. X80301), cystatin proteinase inhibitor PIP-M and PIP-N proteins (K. Salchert, unpubl.). cDNAs coding for carboxy-terminal segments of  $\alpha$ -importin ATHKAP2 were cloned as *Bam*HI-*Xho*I fragments in pGEX-5X-1 (Pharmacia) to isolate GST-ATHKAP2 fusion proteins from *E. coli* by purification on glutathione-S-Sepharose (Ausubel et al. 1989). *PRL1* cDNA was PCR-amplified using a 5'-primer carrying a T7 promoter, sequenced, and used as template to synthesize [<sup>35</sup>S]methionine PRL1 protein using an in vitro coupled transcription and translation kit (Promega). GST and GST-ATHKAP2 proteins were immobilized on glutathione-S-Sepharose, then equal aliquots from the <sup>35</sup>S-labeled *PRL1* protein were incubated with these matrices, as well as with the empty Sepharose matrix, in a binding buffer [20 mM Tris-HCl (pH 7.0), 150 mM NaCl, 2 mM EDTA, and 0.1% NP-40] for 1 hr at 4°C. The matrices were washed four times with binding buffer, then the bound proteins were eluted with 4 $\times$  SDS-sample buffer (Ausubel et al. 1989), and together with the supernatant fractions were size-fractionated on an SDS-PAGE to visualize the labeled PRL1 protein by autoradiography.

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## Pleiotropic control of glucose and hormone responses by PRL1, a nuclear WD protein, in *Arabidopsis*

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