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Overexpression of tomato LeAGP-1 arabinogalactan-protein promotes lateral branching and hampers reproductive development

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Summary

LeAGP-1 is a glycosylphosphatidylinositol (GPI)-anchored arabinogalactan-protein (AGP) in tomato (*Lycopersicon esculentum*). Patterns of mRNA expression and protein localization for LeAGP-1 indicate that it likely functions in certain aspects of plant growth and development. To elucidate LeAGP-1 function(s), transgenic tomato plants expressing enhanced green fluorescent protein (GFP) fused to LeAGP-1 [GFP-LeAGP-1] or two LeAGP-1 variants, one lacking the C-terminal GPI-anchor domain [GFP-LeAGP-1ΔC] and the other lacking the lysine-rich domain [GFP-LeAGP-1ΔK], under the control of the CaMV35S promoter were produced using *Agrobacterium*-mediated transformation. Transgenic T0 and T1 lines with high levels of both GFP-LeAGP-1 mRNA and protein: (i) were significantly shorter; (ii) were highly branched; (iii) produced more flower buds, but most of these flowers did not mature, resulting in less fruit production; and (iv) produced seeds that were significantly smaller than normal seeds. Overexpression of LeAGP-1ΔK had a similar or even more pronounced effect on plant vegetative and reproductive growth, while the effect of LeAGP-1ΔC overexpression on plant reproduction was minimal. These results indicate that the GPI anchor is critical for LeAGP-1 function. As the phenotype of GFP-LeAGP-1 overexpressing transgenic plants is similar to that of cytokinin-overproducing plants, mRNA expression patterns of LeAGP-1 under different hormone treatments were examined. Cytokinins upregulated LeAGP-1 mRNA expression, while auxins and ABA inhibited LeAGP-1 mRNA expression. Based on these results, GPI-anchored LeAGP-1 most likely functions in plant growth and development in concert with auxin/cytokinin signaling.

Keywords: overexpression, LeAGP-1 arabinogalactan-protein, cytokinin, tomato.

Introduction

Arabinogalactan-proteins (AGPs) are hyperglycosylated members of a superfamily of hydroxyproline-rich glycoproteins that are distributed throughout the plant kingdom (Nothnagel, 1997; Showalter, 1993). AGPs can be designated as either 'classical' or 'non-classical' based on the domains and amino acid compositions of the protein backbone (Du *et al.*, 1996). Classical AGPs are predominantly anchored in the plasma membrane with glycosylphosphatidylinositol (GPI)-lipid tails and found in large amounts in the cell wall and extracellular matrix, which likely results from cleavage of plasma membrane AGPs by phosphatidylinositol-specific phospholipase C or D (Oxley and Bacic, 1999; Sherrier *et al.*, 1999; Svetek *et al.*, 1999; Youl *et al.*, 1998). Schultz *et al.* (2002) identified 13 classical AGPs and three basic AGPs with a short Lys-rich subdomain by searching the Arabidopsis genomic database for Pro, Ala, Ser and Thr-rich proteins; all were predicted to be GPI-anchored. Using similar data mining techniques, Borner *et al.* (2002) discovered that over 40% of 210 GPI-anchored proteins identified in Arabidopsis are AGPs or proteins with probable AGP modules. Although the importance of GPI anchors in plants is unclear, these findings provide clues as to how AGPs interact with other cell surface molecules.

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Definitive biological function(s) of any single AGP remains elusive. Much biochemical, immunohistochemical and molecular evidence indicates that AGPs are involved in different aspects of plant growth and development, including cellular differentiation (Knox, 1997; Nothnagel, 1997; Pennell and Roberts, 1990; Pennell *et al.*, 1989, 1991; Showalter, 2001), xylem development (Gao and Showalter, 2000; Schindler *et al.*, 1995; Zhang *et al.*, 2003), somatic embryogenesis (Egertsdotter and von Arnold, 1995; Kreuger and van Holst, 1993, 1995; Van Hengel *et al.*, 2001), cell division/programmed cell death (Chaves *et al.*, 2002; Gao and Showalter, 1999; Serpe and Nothnagel, 1994; Thompson and Knox, 1998), root epidermal cell growth (Ding and Zhu, 1997; Seifert *et al.*, 2002; Willats and Knox, 1996), pollen tube growth (Cheung *et al.*, 1995; Jauh and Lord, 1996; Mollet *et al.*, 2002; Roy *et al.*, 1998; Wu *et al.*, 2000), and hormone signaling (Park *et al.*, 2003; Suzuki *et al.*, 2002; Van Hengel and Roberts, 2003).

Reverse genetics can be a powerful avenue for determining protein function, complementing biochemical approaches. For example, knockout mutants may help to elucidate AGP function, but this research is still in the early stages. Thus, preliminary characterization of several AGP mutants, with the exception of the AGP17 mutant which demonstrates reduced *Agrobacterium* binding, has not identified any obvious phenotypes, perhaps because of genetic redundancy (Nam *et al.*, 1999; Schultz *et al.*, 2002). Several approaches may overcome these difficulties including making double or triple mutants (Halpin *et al.*, 2001), looking for phenotypes under a variety of environmental conditions, and making overexpression-dominant mutants through activation tagging or sense transgenic technology (Nakazawa *et al.*, 2003; Weigel *et al.*, 2000). For plant species without seed stocks of knockout or tag-activated mutants, transgenic approaches involving antisense suppression, overexpression, and RNA interference (RNAi) can be employed to elucidate protein function.

LeAGP-1, a major AGP in tomato, represents one of the most well-characterized AGPs to date. LeAGP-1 has four distinct regions: an N-terminal signal sequence for secretion, a central hydroxyproline/proline-rich region interrupted by a short lysine-rich basic region, and a hydrophobic C-terminal sequence identified as a GPI-anchor addition sequence. Previous work on LeAGP-1 focused on its purification, structure, expression, and immunolocalization (Gao *et al.*, 1999; Li and Showalter, 1996; Sun *et al.*, 2004; Zhao *et al.*, 2002). Availability of the *LeAGP-1* gene and cDNA makes it possible to elucidate LeAGP-1 function through genetic manipulation. Antisense work proved to be an efficient tool in inhibiting expression of many genes and analyzing their functions, but this approach has not provided definitive information in our case. Phenotypes of LeAGP-1 antisense transgenic tomato plant lines are not significantly different from wild-type plants (H. Lu, M. Gao, and A.M. Showalter, unpublished data). In this paper, transgenic tomato plants expressing green fluorescent protein (GFP) fused to LeAGP-1 under the control of the CaMV 35S promoter were generated to assess the function(s) of LeAGP-1. The resulting phenotypes of these overexpression plants provide evidence for LeAGP-1 functioning in specific aspects of vegetative and reproductive growth.

Results

Production of transgenic tomato plants

In order to overexpress LeAGP-1, a chimeric gene consisting of the LeAGP-1 signal sequence (ss)/GFP/sense *LeAGP-1* under the control of the CaMV 35S promoter was introduced into tomato plants through tomato cotyledon transformation. Two mutant gene constructs *GFP-LeAGP-1ΔC* (lacking the C-terminal GPI-anchor domain) and *GFP-LeAGP-1ΔK* (lacking the lysine-rich domain) were also transformed into tomato plants to examine the roles of the GPI lipid anchor and Lys-rich subdomain of LeAGP-1. Transformation of a 35S promoter/ss/GFP construct was used as a control. A total of 30 independent tomato transformants for the 35S-GFP-LeAGP-1, 22 for 35S-GFP-LeAGP-1ΔC, seven for 35S-GFP-LeAGP-1ΔK, and 16 for 35S-GFP constructs were produced. Multiple plants were generated from each callus, but only one was chosen per callus for further analysis. For convenience, transformants with the full-length *LeAGP-1* construct were numbered LE-1, LE-2,…,LE-30; transformants with LeAGP-1ΔC were called ΔC-1, ΔC-2,…,ΔC-22; transformants with LeAGP-1ΔK were named ΔK-1, ΔK-2,…,ΔK-7 and transformants with GFP control were called GFP-1, GFP-2,…,GFP-16. Transformants were examined using PCR to verify presence of the transgene. Twenty-nine LE lines, 18 ΔC lines, five ΔK lines, and 16 GFP lines had the appropriate transgene (data not shown). The 16 GFP lines displayed phenotypes indistinguishable from wild-type (wt) plants, while LE, ΔC and ΔK lines displayed abnormal phenotypes with respect to stem length, lateral branching, fruit number, and seed size.

Characterization of LE transgenic plants via Northern and Western analyses

To correlate phenotypes of the LE line with gene expression, mRNA and protein levels of LeAGP-1 were determined for LE plants at 10 weeks, a time at which phenotypic changes in stem length and lateral branching were noted. Total RNA was isolated from young stems, and expression of endogenous and transgenic *LeAGP-1* RNA determined by Northern blot analyses. Of the 29 LE plants, 21 plants, including LE-9, 11, 12, and 14 showed high levels of *GFP-LeAGP-1* mRNA and normal levels of

endogenous *LeAGP-1* mRNA. Four plants, including LE-15, showed moderate levels of *GFP-LeAGP-1* mRNA and normal levels of endogenous *LeAGP-1* mRNA, and another four plants, including LE-16, showed low levels of *GFP-LeAGP-1* mRNA and endogenous *LeAGP-1* mRNA (Figure 1a). Cosuppression may be responsible for such low mRNA levels in the later group of transgenic plants (Napoli *et al.*, 1990; Van der Krol *et al.*, 1990). To test whether the different *LeAGP-1* mRNA levels could be correlated with LeAGP-1 glycoprotein levels in LE plants, Western blotting was performed (Figure 1b). In LE plants with high *LeAGP-1* mRNA levels (LE-11, 12, 14), LeAGP-1 glycoprotein levels were much higher than GFP-transformed control plants. For LE plants with moderate *LeAGP-1* mRNA levels (LE-15), LeAGP-1 glycoprotein levels were slightly higher than controls. For LE plants with low *LeAGP-1* mRNA levels (LE-16), LeAGP-1 glycoprotein levels were lower than controls. Thus, LeAGP-1 mRNA and glycoprotein levels were directly correlated (Figure 1a,b).

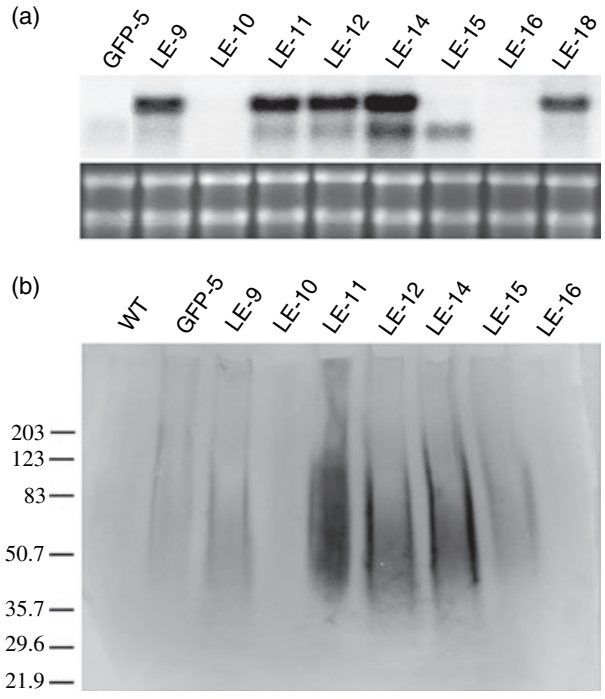

Figure 1. LeAGP-1 mRNA and protein expression in tomato LE transgenic plants.

(a) Northern blot analysis of different LE transgenic plants. Total RNA (10 μg per lane) was separated on a 1% agarose-formaldehyde gel and transferred onto a nylon membrane. *LeAGP-1* cDNA was radioactively labeled as a hybridization probe. Upper panel, the band with large molecular weight indicates transgene expression while the lower band indicates endogenous expression of LeAGP-1. Lower panel, ethidium bromide staining of ribosomal RNA indicates equivalent loading of RNA samples.

(b) Western blot analysis of different LE transgenic plants. Total crude proteins (100 μg per lane) were loaded and separated by SDS-PAGE. An antibody directed against the lysine-rich region of LeAGP-1 (PAP antibody) was used to detect expression of LeAGP-1. Molecular weight markers are shown on the left. Different transgenic lines are shown at the top of each lane.

Overexpression of LeAGP-1 inhibits stem elongation and promotes lateral branching

Phenotypes of all 16 GFP-transformed plants were indistinguishable from wt plants. In contrast, transgenic plants overexpressing LeAGP-1 exhibited several unique phenotypes. One was inhibition of stem elongation, the degree of which was directly correlated with LeAGP-1 mRNA and glycoprotein levels (Figure 2a). LE plants with high LeAGP-1 mRNA and glycoprotein levels (such as LE-11) showed very reduced shoot elongation (Figure 2a and Table 1). Some plants were only one-third as tall as wt or GFP-transformed plants (Table 1). LE plants (such as LE-15) with moderate LeAGP-1 mRNA and glycoprotein levels were about three quarters as tall as wt/GFP control plants. The heights of LE plants with low LeAGP-1 mRNA and glycoprotein levels were indistinguishable from control plants. (Figure 2a and Table 1).

In addition to reduced stem elongation, LeAGP-1-overexpressing transgenic plants were highly branched, having about twice as many lateral branches as wt and GFP-transformed plants (Table 1). As with plant height, the number of lateral branches in LE plants was directly correlated with LeAGP-1 expression. The number of lateral branches in LE plants with high levels of LeAGP-1 expression was about twice as great as that of GFP-transformed plants, while LE plants with low levels of LeAGP-1 expression had about an equal number of lateral branches compared with controls (Table 1).

Overexpression of LeAGP-1 reduces fruit production and hampers seed development

In addition to the effect on vegetative growth, LeAGP-1 overexpression affected plant reproduction. LE plants with high levels of LeAGP-1 mRNA and protein produced less fruit than wt plants (Table 1). Although these transgenic LE lines produced more inflorescences than control plants, most of the resulting flower buds never develop completely. LeAGP-1 overexpressors had another notable phenotype; they produced few, normal size seeds. Instead, although they produced the same number of seeds per fruit as control plants, almost all of these seeds were tiny (Figure 2c and Table 1). The weight of these seeds was about 30% of control seeds. Moreover, fruit yield and seed size in the transgenic plants correlated with the expression level of LeAGP-1.

Identification of transgenic plants with LeAGP-1ΔC and LeAGP-1ΔK overexpression

Northern blot analyses showed that three of the five ΔK lines had high levels of transgenic and normal levels of endogenous *LeAGP-1* mRNA, one line (ΔK-5) had moderate levels

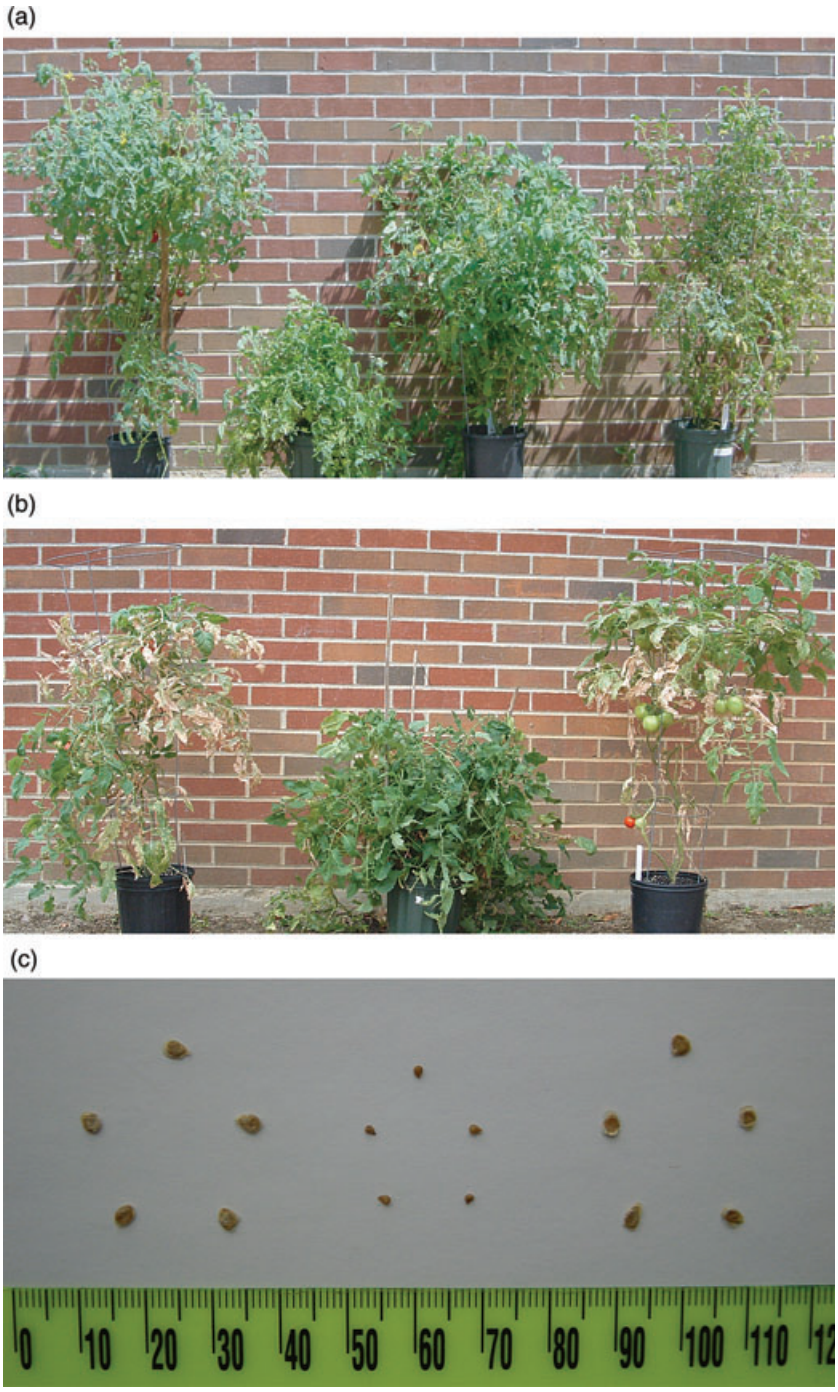

Figure 2. Phenotypes of tomato LE transgenic plants.
(a) The phenotypes of tomato transgenic plants with different levels of LeAGP-1 expression. From left to right: GFP-5 (control), LE-11 (high expression of LeAGP-1), LE-15 (moderate expression of LeAGP-1), LE-16 (low expression of LeAGP-1).
(b) The phenotype of T1 progeny of LE-14 (high expression of LeAGP-1). From left to right: wild-type plant, LE-14.15 (LeAGP-1 overexpression), LE-14.5 (low expression of LeAGP-1).
(c) The seed phenotype of tomato transgenic plants. From left to right: GFP-5 (control), LE-14 (high expression of GFP-LeAGP-1), ΔC-5 (high expression of GFP-LeAGP-1ΔC mRNA).

of transgenic and normal levels of endogenous *LeAGP-1* mRNA, and another line (ΔK-2) had very low levels of both transgenic and endogenous *LeAGP-1* mRNA (Figure 3a). Transgenic plants with LeAGP-1ΔK overexpression had similar phenotypes to LeAGP-1-overexpressing LE lines, namely, dwarf, bushy plants, producing less fruit and small seeds (Table 2). The only differences between them were the more dramatic effect that LeAGP-1ΔK overexpression had on further reducing both fruit production and the number of normal-sized seeds.

The ΔC transgenic plants were also categorized into three groups based on Northern blot analyses – high, moderate and low LeAGP-1ΔC expressors (Figure 3b). Transgenic plants with LeAGP-1ΔC overexpression also demonstrated a dwarf, bushy phenotype. However, the effect of LeAGP-1ΔC overexpression on fruit production

Table 1 Phenotypes of different tomato transgenic plants transformed with *35S-GFP-LeAGP-1*

Lines[a]	Plant height (cm)[b]	Branch no.[b]	Fruit no.[c]	Normal seeds (%)[d]
WT	80	45	121	67.8
GFP-5(C)	84	45	110	62.0
LE-9(H)	40	78	23	1.5
LE-10(L)[e]	62	29	11	36.4
LE-11(H)	28	90	13	9.5
LE-12(H)	28	82	24	0.8
LE-14(H)	46	85	44	0.8
LE-15(M)	58	50	66	34.2
LE-16(L)	79	39	106	53.9
LE-18(H)	39	109	33	0

[a]LeAGP-1 expression levels are indicated in parenthesis as H for high, M for moderate, L for low, and C for control.
[b]Plant heights and branches were counted when plants were 6 months old.
[c]Fruits were collected throughout the growth period (18 months).
[d]The percentage of normal seeds was averaged from at least 10 fruits.
[e]LE-10 died after approximately 5 months.

and seed development was minimal. No significant differences in fruit yield and seed development existed among the ΔC lines compared with control plants with no transgene (Table 2, Figure 2c).

Further identification of phenotypes in T1 generation of transgenic plants

Several LE transgenic lines with LeAGP-1 overexpression were selected for further analyses in their T1 generation. Mutant phenotypes also appeared in T1 progeny. Among these, T1 progeny of the LE-14 line were examined by PCR, Northern blotting, Western blotting, and growth measurements (Figures 2b and 4). Of 20 plants, 16 were confirmed to have the transgene. Combined with kanamycin resistance results (data not shown), these data indicate that the LE-14 transgenic line had a single T-DNA insertion site. Ten of these T1 plants had high expression of the transgene as determined by Northern blot analysis (Figure 4a), and Western blotting indicated that the LeAGP-1 protein level was directly correlated with *LeAGP-1* mRNA expression (Figure 4b). Interestingly, stunting of stem elongation in T1 LeAGP-1 overexpressors occurred largely in later stages of growth (Figure 2b). No significant differences were detected between transgenic and control plants during the first 6-week growth period, but growth differences gradually became obvious thereafter (Figure 5). Similarly, enhanced lateral branching in LeAGP-1-overexpressing T1 transgenic plants occurred in later stages of growth (data not shown). In addition, T1 LeAGP-1 overexpressors displayed delayed leaf senescence, compared with wt plants at the same growth stage (Figure 2b). Delayed leaf senescence was also analyzed and verified by measuring enhanced chlorophyll content in LeAGP-1 overexpressors compared with GFP control plants (Figure S1).

LeAGP-1 mRNA expression is regulated by plant hormones in tomato seedlings

Overexpression of LeAGP-1 in tomato plants stunted elongation of the main stem axis and promoted lateral shoot development, leading to bushy plants. These morphological phenotypes were remarkably similar to those of cytokinin-overproducing or auxin-underproducing plants (Cline, 1994; Li *et al.*, 1992). This indicated that the functions of LeAGP-1 in plant growth and development might be associated with the balance of auxin and cytokinin. To test this hypothesis, *LeAGP-1* mRNA expression patterns, following treatment with various types and concentrations of plant hormones, were determined. The results showed that auxins [2, 4-chlorophenoxyacetic acid (2,4-D) and 4-chlorophenoxyacetic acid (CPA)] downregulated LeAGP-1 expression and cytokinins (zeatin and kinetin) upregulated

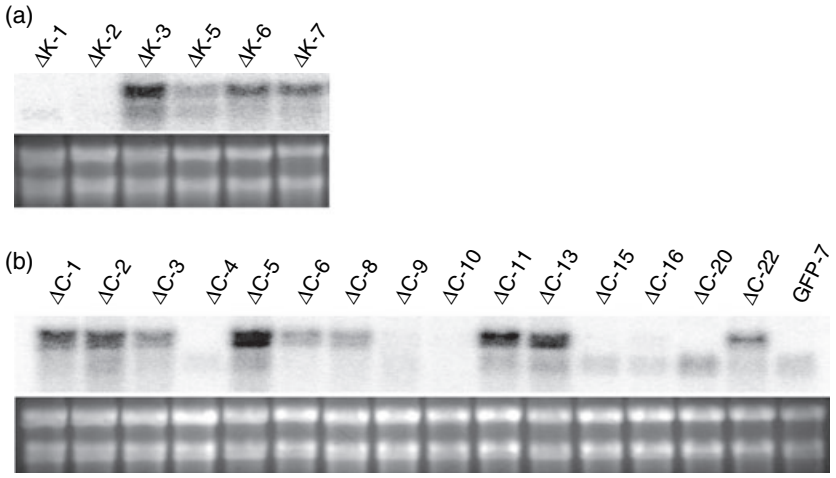

Figure 3. *LeAGP-1* mRNA in LeAGP-1 deletion mutants in tomato ΔK and ΔC transgenic lines.
(a) Northern blotting analysis of different ΔK transgenic plants. ΔK-1 was identified to have no transgene via PCR (as a control).
(b) Northern blot analysis of different ΔC transgenic plants. Total RNA (10 μg per lane) was separated on a 1% agarose-formaldehyde gel and transferred onto a nylon membrane. *LeAGP-1* cDNA was radioactively labeled as a hybridization probe. Upper panel, the band with large molecular weight indicates transgene expression while the lower band indicates endogenous expression of LeAGP-1. Lower panel, ethidium bromide staining of ribosomal RNA showing equivalent loading of RNA samples. Different transgenic lines are indicated at the top of each lane.

Table 2 Phenotypes of different tomato transgenic plants transformed with *35S-GFP-LeAGP-1ΔK* and *35S-GFP-LeAGP-1ΔC*

Lines[a]	Plant height (cm)[b]	Branch no.[b]	Fruit no.[c]	Normal seeds (%)[d]
ΔK-1(C)[e]	76	42	19	55.5
ΔK-2(L)	79	47	37	63.7
ΔK-3(H)	43	164	3	0
ΔK-5(M)	61	56	19	24.4
ΔK-6(H)	48	73	0	nd
ΔK-7(H)	49	78	0	nd
ΔC-1(H)	64	79	41	69.3
ΔC-3(H)	71	92	47	66.9
ΔC-4(C)[e]	84	42	34	67.6
ΔC-5(H)	54	70	22	55.0
ΔC-9 (M)	78	68	40	70.2
ΔC-10(L)	77	42	31	68.7
ΔC-11(H)	62	73	42	75.1
ΔC-15(N)	82	43	54	69.2

nd, not determined.
[a]LeAGP-1 expression levels are indicated in parenthesis as H for high, M for moderate, L for low, and C for control.
[b]Plant heights and branches were counted when plants were 6 months old.
[c]Fruits were collected throughout the growth period (9 months).
[d]The percentage of normal seeds was averaged from at least 10 fruits, if available.
[e]ΔK-1 and ΔC-4 were determined to have no transgenes by PCR, and were used as controls.

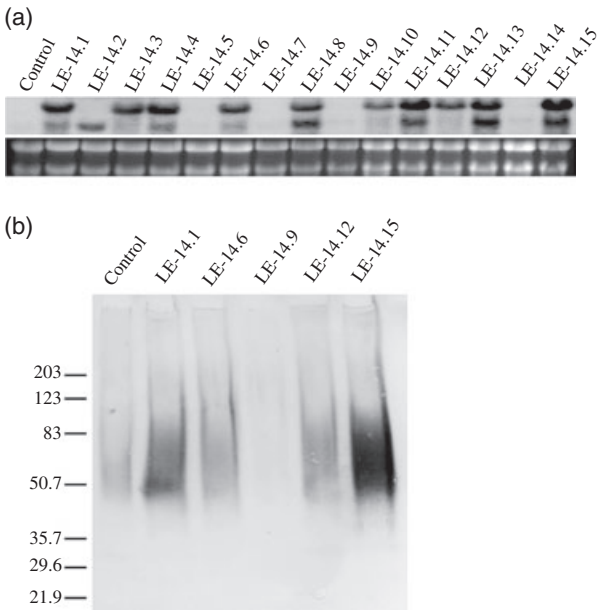

Figure 4. LeAGP-1 mRNA and protein expression in tomato T1 progeny of transgenic line LE-14.
(a) Northern blot analysis of different transgenic plants. Total RNA (10 μg per lane) was separated on a 1% agarose-formaldehyde gel and transferred onto a nylon membrane. *LeAGP-1* cDNA was radioactively labeled as a hybridization probe. In the upper panel, the band with the large molecular weight indicates transgene expression while the lower band indicates endogenous expression of *LeAGP-1*. In the lower panel, ethidium bromide staining of ribosomal RNA shows equivalent loading of RNA samples.
(b) Western blot analysis of different T1 transgenic plants. Total crude proteins (100 μg per lane) were loaded and separated via SDS-PAGE. Different transgenic lines as indicated were examined with the PAP antibody to detect expression of LeAGP-1. Molecular weight markers are shown on the left. Different transgenic lines are indicated at the top of each lane.

LeAGP-1 expression in tomato seedlings (Figure 6a). In addition, abscisic acid (ABA) markedly inhibited *LeAGP-1* mRNA expression in tomato seedlings. Furthermore, the promoting/inhibiting effects of zeatin, 2, 4-D and ABA on *LeAGP-1* mRNA expression were concentration-dependent (Figure 6b).

Discussion

AGPs are implicated in many different aspects of plant growth and development; however, definitive biological functions for any single AGP remain uncertain. In an effort to understand the role of LeAGP-1 in plant growth and development, transgenic tomato plants overexpressing GFP-LeAGP-1 were produced and referred to as LE lines. This work represents an outgrowth of related studies performed in cell cultures (Sun *et al.*, 2004; Zhao *et al.*, 2002). In those studies, GFP tagging of LeAGP-1 did not interfere with post-translational modification of LeAGP-1 or with its targeting. Here, compared with wt and GFP control plants, LE lines overexpressing LeAGP-1 demonstrated: (i) less stem elongation, (ii) enhanced lateral branching, (iii) less fruit production, and (iv) small, viable seeds (Figure 2 and Table 1). These effects on vegetative growth and reproduction are directly correlated with LeAGP-1 protein expression levels (Figure 2a), although the unlikely possibility that GFP in the fusion protein contributes to these effects cannot be excluded. Moreover, these effects on vegetative growth are insignificant at early stages of seedling development, but become pronounced after 6 weeks of growth. To evaluate the importance of the GPI anchor and lysine-rich domain in LeAGP-1 function, two deletion mutants of LeAGP-1, GFP-LeAGP-1ΔC and GFP-LeAGP-1ΔK, were overexpressed in transgenic tomato plants. Phenotypes of ΔK transgenic lines were quantitatively, but not qualitatively different from those of LE transgenic plants. In contrast, phenotypes of ΔC transgenic lines were different from those of LE plants with respect to plant reproduction. Fruit yield and seed development in ΔC plants were not significantly different from control plants, which likely relates to the observation that these plants, unlike LE overexpressors, exhibit normal leaf senescence and concomitant remobilization of nutrients for fruit set. Thus, the GPI anchor is important for LeAGP-1 function with respect to these aspects of plant reproduction.

Notably, cosuppressed transgenic plants display no observable phenotypes compared with wt plants. This

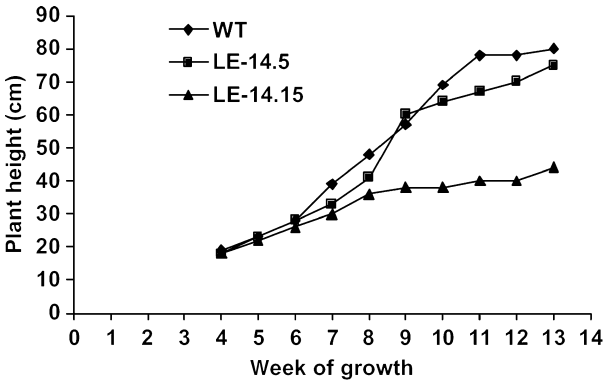

Figure 5. Inhibition of stem elongation by LeAGP-1 overexpression. For 6 weeks following germination, there are no differences in height of WT, LE-14.5 (low LeAGP-1 expression), and LE-14.15 (high LeAGP-1 expression). Differences, however, were gradually observed after 6 weeks, as indicated by the growth curve of total plant height (cm).

finding is consistent with results of phenotypic screens of AGP mutants in Arabidopsis which fail to identify differences from wild type (Schultz *et al.*, 2002). Genetic redundancy within the AGP gene family may account for these observations. Alternatively, analysis of such mutants may not be sufficiently rigorous or sensitive to reveal phenotypes that are hidden unless a wide variety of environmental and/or stress conditions are utilized (Boyes *et al.*, 2001; Meissner *et al.*, 1999).

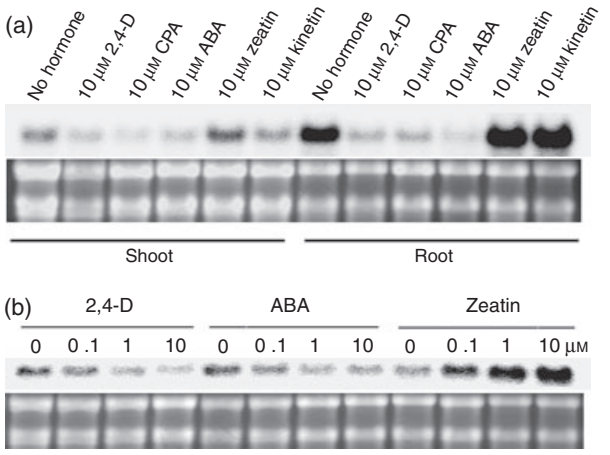

Figure 6. *LeAGP-1* mRNA expression in tomato seedlings regulated by plant hormones.
(a) *LeAGP-1* mRNA expression was downregulated by 10 μM 2,4-D, CPA, ABA, but was upregulated by 10 μM zeatin and kinetin. No hormone treatment was used as a control. Lanes 1–6, 20 μg total RNA isolated from shoots of tomato seedlings and lanes 7 –12, 15 μg total RNA isolated from roots of tomato seedlings.
(b) Regulation of *LeAGP-1* mRNA expression in tomato seedlings by 2,4-D, ABA, and zeatin was concentration-dependent. Different concentrations and kinds of phytohormones are indicated above each lane. Upper panel, the band indicates *LeAGP-1* mRNA expression; lower panel, ethidium bromide staining of ribosomal RNA shows equivalent loading of RNA samples.

Cytokinins are an important class of plant growth regulators. Cytokinins play critical roles in many plant developmental processes, such as cell division, cell differentiation, leaf senescence, and apical dominance (Mok and Mok, 1994). Transgenic tobacco plants overexpressing isopentenyl transferase from *Agrobacterium tumefaciens* increase their cytokinin content and become bushy (Li *et al.*, 1992; Medford *et al.*, 1989). Here, tomato LeAGP-1 overexpressors have a similar bushy phenotype. A study of the T1 progeny of LE-14 showed that mutant phenotypes were stable and inheritable. While delayed leaf senescence in T1 LeAGP-1 overexpressors is consistent with the function of cytokinins in the development of chlorophyll (Figure 2b and Figure S1), anything that delays or reduces fruit set tends to have a major impact on senescence. Given the reduced fruit set of the overexpressors, this effect on senescence may be indirect. The observed phenotypes resulting from LeAGP-1 overexpression indicate that LeAGP-1 might function in concert with cytokinin and/or auxin signal transduction pathways. Furthermore, regulation of *LeAGP-1* mRNA expression by auxins and cytokinins corroborates this hypothesis.

AGPs are implicated in a variety of cellular and developmental processes such as cell division, cell expansion, and plant differentiation (reviewed in Nothnagel, 1997; Showalter, 2001), which are all regulated by plant hormones. β-Yariv reagent inhibited gibberellin-induced α-amylase production, indicating that AGPs are involved in gibberellin signal transduction (Suzuki *et al.*, 2002). Moreover, AGPs can increase cell division of carrot protoplasts similar to auxin (Van Hengel *et al.*, 2001). These observations indicate a relationship between AGPs and plant hormone signal transduction pathways.

Additionally, ABA significantly inhibits *LeAGP-1* mRNA expression in tomato seedlings (Figure 6) and is consistent with our finding that the *LeAGP-1* gene contains GA and ABA-responsive elements (e.g. TCTTTT elements) (Gubler and Jacobsen, 1992; Sutoh and Yamauchi, 2003). ABA is a small, lipophilic plant hormone that regulates plant development, seed dormancy, germination, cell division, and cellular responses to environmental stresses such as drought, cold, salt, pathogen attack, and UV radiation (reviewed by Rock, 2000). In seeds, ABA levels peak during late embryogenesis when storage proteins and nutrient reserves accumulate (Rock and Quatrano, 1995). The small, viable seeds produced by LeAGP-1-overexpressing transgenic plants may result from limited production and deposition of storage proteins and nutrient reserves, indicating LeAGP-1 might act as a negative mediator of ABA signaling. In this context it is interesting to note that a plasma membrane glycoprotein in rice protoplasts recognized by JIM 19 is involved in ABA signaling (Desikan *et al.*, 1999). AtAGP30, a non-classical AGP in primary root cell walls, was suggested to function in ABA signaling (Van Hengel and

Roberts, 2003). Furthermore, ABA levels increased two- to 50-fold in tomato leaves 6 h after wounding (Herde *et al.*, 1996; Pena-Cortes *et al.*, 1989, 1996). This report is consistent with the observation that mRNA expression of *LeAGP-1* and its ortholog, *NaAGP4*, were inhibited after wounding and pathogen attack (Gilson *et al.*, 2001; Li and Showalter, 1996).

One recent seminal finding in AGP research is their association with GPI lipid anchors. A bioinformatics algorithm was designed and used to mine the Arabidopsis database for potential GPI-anchored proteins (GAPs). Forty percent of the predicted 248 GAPs in Arabidopsis are AGPs or proteins with AGP glycomodules, and represent major components of the plasma membrane (Borner *et al.*, 2002, 2003; Sherrier *et al.*, 1999). The importance of GPI anchors is not yet clear in plants. In many organisms, GPI anchor structures are implicated in: (i) regulated release from cell surfaces by phospholipase C or D, (ii) polarized attachment to different regions of cell surfaces, (iii) increased lateral mobility in the lipid layer, (iv) association with lipid microdomains, (v) recycling from the plasma membrane, and (vi) 'protein painting,' a process of GAPs transferring from one cell surface to the plasma membrane of an adjacent cell (Brown, 2002; Brown *et al.*, 2000; Fivaz *et al.*, 2002; Hooper, 1997; Muniz and Riezman, 2000; Premkumar *et al.*, 2001). In this research, comparison of the phenotypes between LeAGP-1 and LeAGP-1ΔC-overexpressing transgenic plants indicated that the GPI anchor plays an important role in LeAGP-1 function with respect to fruit production and seed development.

Two models relating LeAGP-1, plant hormones and plant growth are plausible. In one model, LeAGP-1 functions downstream of plant hormone signal transduction. Specifically, we propose: (i) that cytokinins promote LeAGP-1 expression in plants, consistent with cytokinin-induced *LeAGP-1* mRNA accumulation (Figure 6) and with our bioinformatics analysis of the *LeAGP-1* gene which revealed two copies of the as-1 TGACG motif which is found in cytokinin responsive genes (Jin *et al.*, 1998 and Yang *et al.*, 2002); and (ii) that the increased amount of LeAGP-1 glycoprotein then directly functions to stimulate cell division/proliferation, resulting in enhanced lateral branching and concomitant suppression of apical growth. Previous research has demonstrated that AGPs are involved in cell division (reviewed in Nothnagel, 1997). Experimental results with *Gymnocolea inflata* indicate that AGPs mediate plant morphogenesis/organogenesis (Basile and Basile, 1993). Interestingly, OVE mutants of *Physcomitrella patens* Hedw., gametophore-overproducing mutants, developed similar phenotypes to wt strains cultured in the presence of high levels of cytokinins (Ashton *et al.*, 1979, 1993; Cove *et al.*, 1980) and produced significantly more AGPs than wt strains (Mignone and Basile, 2000). These results are consistent with our observations.

In the second model, LeAGP-1 functions upstream of the plant hormone signaling pathway. This model is mainly based on the signaling role that AGP analogs (i.e. certain proteoglycans) play in animals. Two major families of cell surface heparan sulfate proteoglycans exist in animals, syndecans and glypicans. Like classical AGPs in plants, glypicans are linked to the plasma membrane via GPI anchors. These proteoglycans play critical roles in several major developmental signaling pathways involving animal growth factors by serving as co-receptors (De Cat and David, 2001; Perrimon and Bernfield, 2000). Specifically, glycosaminoglycans of glypicans bind growth factors, extracellular matrix molecules, enzymes, protease inhibitors and other proteins, creating areas of concentrated ligands (Lindahl *et al.*, 1998). These high-affinity glypicans co-receptors then pass on the ligands to low-affinity growth factor receptors (De Cat and David, 2001; Zhang *et al.*, 2001). By analogy, LeAGP-1 may serve as a regulator or co-receptor for plant hormone signaling. The carbohydrate moiety of LeAGP-1 anchored to the plasma membrane may have affinity to plant growth factors, such as cytokinins, and collects these growth factors on the cell surface, facilitating interaction of the hormone and its receptor on the plasma membrane. Alternatively, GPI-anchored LeAGP-1 might function as a plant hormone carrier and transport growth factors inside the cell when the plasma membrane is internalized, analogous to glypican-mediated uptake of polyamines (Fransson, 2003). While further experimentation is required to test these models and integrate LeAGP-1 into a signal transduction pathway, the significance of this research in assigning specific functional roles for LeAGP-1 in vegetative and reproductive growth is clear and complements the substantial amount of structural information on this particular AGP.

Experimental procedures

Overexpression constructs

A CaMV 35S promoter/LeAGP-1 signal sequence(ss)/*GFP*/sense *LeAGP-1* chimeric gene construct and its two deletion mutants, 35S promoter /ss/*GFP*/*LeAGP-1* lacking the GPI-anchor (*GFP-LeAGP-1ΔC*) and 35S promoter/ss/*GFP*/*LeAGP-1* lacking the lysine-rich region (*GFP-LeAGP-1ΔK*), were produced as described previously (Sun *et al.*, 2004; Zhao *et al.*, 2002). Another construct 35S promoter/ss/*GFP* was also transformed into tomato plants as a control.

Agrobacterium *and tomato cotyledons transformation with four different constructs*

The pBI121-based plasmids containing the *GFP-LeAGP-1* constructs and *GFP* control construct were transformed into *A. tumefaciens* strain LBA4404 by the freeze-thaw method (An *et al.*, 1988). *Agrobacterium*-mediated transformation and regeneration of tomato (*Lycopersicon esculentum*) was carried out according to McCormick

(1991) with minor modifications. Briefly, tomato (*L. esculentum* cv. UC82B) seeds were germinated on 1/2 × MSO media in magenta boxes after sterilizing in 20% household bleach and 0.1% Tween-80 for 15 min. Seven- to 10-day-old cotyledons were sliced and placed upside down on petri dishes containing D1 medium [4.3 g l^{-1} MS basic salts, 3% (w/v) glucose, 1 × Gamborg's B5 vitamins, 1 mg l^{-1} zeatin, 0.8% (w/v) agar, pH 5.8]. Sliced cotyledons were co-incubated with 2-day-old *Agrobacterium* cultures for 2 days at 26°C under a 16 h light/8 h dark cycle. Afterward, cotyledons were transferred to selection plates containing D1 medium, 400 mg l^{-1} Timentin, and 100 mg l^{-1} kanamycin for 4 weeks under the same growth conditions as above. Cotyledons with calli and/or tiny shoots were transferred to plates containing D2 medium [4.3 g l^{-1} MS basic salts, 3% (w/v) glucose, 1 × Gamborg's B5 vitamins, 0.1 mg l^{-1} zeatin, 0.8% (w/v) agar, pH5.8], 400 mg l^{-1} Timentin, and 100 mg l^{-1} kanamycin for further shooting. Shoots with true meristems were transferred to rooting medium [4.3 g l^{-1} MS basic salts, 3% (w/v) glucose, 1 × Gamborg's B5 vitamins, 0.8% (w/v) agar, pH 5.8], 400 mg l^{-1} Timentin, and 100 mg l^{-1} kanamycin for 2–3 weeks. Plantlets were transferred into soil pots within magenta boxes and grown for several days before transfer to the greenhouse.

Identification of transgenes using PCR

Four oligonucleotide primers were designed and synthesized by Integrated DNA Technologies (Coralville, IA, USA). The first one 5′-GGA CGA CGG CAA CTA CAA G-3′ lies in the 5′-terminus of the *GFP* cDNA, the other three were: 5′-CGG TCA CGA ACT CCA GCA-3′, 5′-GTG TTT CTT TCC CTT CTT TCC C-3′, and 5′-ACC AAA CTT CCC AGC ATC TTC-3′, which respectively complemented sequences in the *GFP* cDNA (3′ end), lysine-rich domain, and C-terminal GPI anchor domain. DNA was isolated from transgenic and wt plants using Plant DNAzol Reagent (Invitrogen, Carlsbad, CA, USA). Polymerase chain reactions were performed according to standard protocols.

Northern blotting

Total RNA was extracted from young (10-week-old) stems of transgenic plants harboring the four different genetic constructions, as well as from wt plants using the RNeasy Plant Total RNA kit (Qiagen, Chatsworth, CA, USA). Total RNA was electrophoresed in 1% agarose-formaldehyde gels and transferred by capillary action onto Zeta-Probe GT Genomic Tested Blotting Membranes (Bio-Rad, Hercules, CA, USA). *LeAGP-1* and *GFP* probes were labeled with α-^{32} P-dCTP using the Prime-a-gene labeling system (Promega, Madison, WI, USA). Prehybridizations and hybridizations were performed according to instructions provided by the membrane supplier. Equivalent loading of RNA samples was confirmed by ethidium bromide staining of ribosomal RNA.

Western blotting

Young (10-week-old) stems from the different transgenic plants were ground with a mortar and pestle in liquid nitrogen and then homogenized in 100 mM $AlCl_3$ before centrifugation for 20 min at 10 000 ***g***. Supernatants were dialyzed against distilled H_2O at 4°C for 2 days and lyophilized. Quantification of proteins was accomplished with a Bio-Rad DC protein assay kit II (Bio-Rad). Western blotting was conducted as described previously using the LeAGP-1-specific polyclonal antibody (PAP antibody) (Gao *et al.*, 1999; Sun *et al.*, 2004).

Chlorophyll extraction and analysis

Young leaves were detached from transgenic and control plants and incubated in a petri dish with dH_2O. Chlorophyll was extracted from leaves at 0, 2, 4, and 6 days post-detachment following the procedure of Wintermans and De Mots (1965). Briefly, three leaves (0.1–0.2 g) were ground in liquid nitrogen to fine powder using a mortar and pestle. The powder was transferred into centrifuge tubes with 1.5 ml 90% ethanol. Cell debris was removed by centrifugation at 16 600 ***g*** at 4°C for 5 min. The supernatant (333 μl) was diluted up to 1.5 ml with ethanol and OD649, OD665, and OD750 were recorded using a spectrophotometer. Chlorophyll content was calculated using the equation: chlorophyll $(a + b) = 6.10$ (OD665 – OD750) + (OD649 – OD750).

Plant hormone treatment of tomato seedlings

Tomato seeds were sterilized and germinated on MSO medium; 7-day-old seedlings were transferred onto MSO medium with different kinds and concentrations of plant hormones as described. After 2 days of treatment, seedlings were collected for RNA isolation.

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Supplementary Material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/TPJ/TPJ2274/TPJ2274sm.htm

Figure S1. Delayed loss of chlorophyll in GFP-LeAGP-1 overexpressors (LE-14 and LE-19) compared with GFP control plants (GFP9). Values are mean ± SE of three independent experiments (F. Wt, fresh weight).

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