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Functional analysis of the BET gene GTE4

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The Arabidopsis BET bromodomain Factor GTE4 is Involved in Maintenance of the Mitotic Cell Cycle During Plant Development

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ABSTRACT
BET proteins are characterized by the presence of two types of domains, the bromodomain and the extra terminal (ET) domain. They bind to acetylated lysines present on histone tails and control gene transcription. They are also well known to play an important role in cell cycle regulation. In Arabidopsis there are 12 BET genes, however only two of them, IMBITION INDUCIBLE 1 (IMB1) and GENERAL TRANSCRIPTION FACTOR GROUP E6 (GTE6) were functionally analysed. We characterized GTE4 and show that gte4 mutant plants have some characteristic features of cell cycle mutants. Their size is reduced, they have jagged leaves and reduced number of cells in most organs. Moreover, cell size is considerably increased in the root, and, interestingly, the root quiescent center identity seems to be partially lost. Cell cycle analyses revealed that there is a delay in activation of the cell cycle during germination and a premature arrest of cell proliferation with a switch from mitosis to endocycling leading to a statistically significant increase in ploidy levels in the differentiated organs of gte4 plants. Our results point to a role of GTE4 in cell cycle regulation and specifically in the maintenance of the mitotic cell cycle.
INTRODUCTION
The histones H2A, H2B, H3 and H4 are the four core proteins that built nucleosomes (Hansen, 2002). They can all be modified by: acetylation, methylation, phosphorylation and ubiquitination and these post translational modifications provide in a combinatorial or sequential fashion the histone code that can dictate specific cellular processes (Strahl and Allis, 2000; Turner, 2000; Zhang and Reinberg, 2001; Eberharter and Becker, 2002; Sun and Allis, 2002; Nowak and Corces, 2004; Bode and Dong, 2005). It has been demonstrated that some of the modifications, especially acetylations, of histone tails are able to relax the packing of the DNA facilitating the access to DNA of many regulatory proteins involved in replication, transcription, repair and recombination (Lorch et al., 1999; Wolff, 2001; de la Cruz et al., 2005).

Proteins containing bromodomains have the important role of deciphering the histone acetylation codes since bromodomains bind acetylated lysines on histone tails (Dhalluin et al., 1999; Jacobson et al., 2000; Strahl and Allis, 2000; Dey et al., 2003; Liu et al., 2008). The bromodomain was first discovered in the Drosophila Brahma protein (Kennison and Tamkun, 1988; Tamkun et al., 1992) and is present in a broad range of chromatin-modifying proteins (Haynes et al., 1992; Jeanmougin et al., 1997; Jacobson et al., 2000; Syntichaki et al., 2000; Dyson et al., 2001; Horn and Peterson, 2001; Schwanbeck et al., 2004; Yang, 2004). BET (bromodomain and extraterminal) proteins form a separate group of bromodomain proteins which all share besides the N-terminal bromodomain(s) an extra terminal (ET) domain (Haynes et al., 1992; Lygerou et al., 1994; Pandey et al., 2002). The ET domain consists of three separate regions, of which only the N-terminal ET (NET) domain is conserved in all BET proteins. The ET domain was shown to have serine-kinase activity and it functions as interaction domain to recruit other proteins or complexes to acetylated histones (Platt et al., 1999).

The first BET family member that was functionally analysed is female sterile homeotic (fsh) of Drosophila (Huang and Dawid, 1990; Chang et al., 2007). This gene has been shown to activate the ULTRABITORAX gene, a homeotic gene involved in Drosophila embryo development. Other well characterised members are the mammalian BET proteins Brd2 and Brd4 (formally RING3 and MCAP) for which it has been demonstrated that they bind to acetylated histones (Dey et al., 2003; Kanno et al., 2004). The same has been observed for their yeast homolog Bromodomain factor 1 (Bdf1) (Pamblanco et al., 2001). Brd2 and Brd4 are expressed in proliferating cells and are fundamental for cell cycle progression (Dey et al., 2000; Houzelstein et al., 2002; Maruyama et al., 2002; Shang et al., 2004; Wu and Chiang, 2007; Mochizuki et al., 2008; Yang et al., 2008). BET proteins identified in yeast are also involved in regulation of the cell cycle. The yeast bdf1 mutant is defective in meiosis and it was also shown to be associated with mitotic chromosomes. Also Brd2...
and Brd4 bind to mitotic chromosomes and binding persists during mitosis (Platt et al., 1999; Dey et al., 2003). It has been hypothesized that these proteins contribute to the transmission of the transcriptional memory from one generation of cells to the next (Matangkasombut et al., 2000; Dey et al., 2003).

Although BET proteins were first identified and mostly studied in yeast, human, mouse and Drosophila, recently BET proteins have also been identified in plants. All plant BET proteins are different from those of yeast and animals since they have only one bromodomain instead of two (Florence and Faller, 2001; Pandey et al., 2002). In animals the presence of two bromodomains seems to be fundamental for their function. However in yeast genetic analysis suggests that the function of Bdf1 in sporulation is only dependent on one bromodomain (Chua and Roeder, 1995). The presence of only one bromodomain in the plant BET protein members was supposed to be compensated by a dimerisation event but there is still no evidence that can support this hypothesis (Florence and Faller, 2001). Twelve BET encoding genes have been identified in the Arabidopsis genome. Until now only two of these have been functionally characterized, IMBIBITION INDUCIBLE 1 (IMB1) and GENERAL TRANSCRIPTION FACTOR GROUP E6 (GTE6). IMB1 plays a role in the promotion of seed germination by regulating negatively the abscisic acid (ABA) pathway and positively the phytochrome transduction pathway (Duque and Chua, 2003). Microarray analysis of the imb1 mutant showed that IMB1 acts predominantly as a transcriptional activator and it is responsible for the activation of genes involved in cell-wall metabolism and plastid-encoded genes. The other characterised plant BET encoding gene GTE6 is involved in the establishment of elliptical leaf shape in mature leaves (Chua et al., 2005). GTE6 was shown to positively regulate the myb-domain gene ASYMMETRIC LEAF 1 (ASI), which is involved in leaf axis specification in mature leaves. It is associated with the promoter and the start of the transcribed region of ASI and up-regulates ASI expression through acetylation of histones H3 and H4.

Although both IMB1 and GTE6 seem to have a role in transcriptional regulation, for none of the two there is evidence for a function in cell cycle regulation.

Here we present the functional analysis of GTE4, a member of a so far uncharacterised group of Arabidopsis BET proteins. The gte4 mutant shows defects during root, leaf and flower development. Cell numbers are significantly reduced in most of the organs. Cell cycle re-activation is delayed in germinating seeds and a premature switch from mitosis to endoreduplication occurs. Furthermore, a partial loss of root quiescent center (QC) identity is observed. The results that we obtained show that GTE4 is involved in the activation and maintenance of cell divisions in the meristems and by this controlling cell numbers in most organs.
RESULTS

**GTE4 encodes a BET bromodomain protein and is expressed in all organs**

The analysis of the Arabidopsis genome sequence allowed the identification of twelve BET proteins. We produced a rooted tree for these Arabidopsis BET proteins using the bromodomain and the ET domain. This analysis supports the previous results obtained by Florence and Faller (2001), since it shows that Arabidopsis BET proteins can be divided into 3 groups which clearly reflect the differences in the entire protein sequence of these Arabidopsis BET proteins. Both IMB1 (GTE1) and GTE6, the first two bromodomain genes that were studied in detail, belong to the same group, whereas GTE4 belongs to a new group of uncharacterized genes (Fig. 1).

To analyse the expression of GTE4, RNA was extracted from roots, rosette leaves, stems, flowers and siliques and analysed by RT-PCR. This analysis revealed that GTE4 is expressed in all tissues (Supplemental Fig. S1).

**GTE4 is not able to form homodimers in a yeast two-hybrid assay**

Plant BET bromodomain proteins have only one bromodomain instead of two. The presence of only one bromodomain was supposed to be overcome by the capacity of plant BET proteins to form dimers (Florence and Faller, 2001). To address if GTE4 could form homodimers we performed a yeast two-hybrid analysis. For this purpose we cloned the GTE4 open reading frame in two different vectors creating fusion proteins with the GAL4 activation (AD) and the binding (BD) domain. We tested at 21°C and 29°C for interaction by selecting yeast double transformants on media lacking histidine to which we added increasing concentrations of 3-amino-1,2,4-triazole and we also tested for interactions using the ß-galactosidase assay. Whereas all positive controls using interacting MADS-box proteins (de Folter et al., 2005) were positive, all the assays in which we tested homodimerisation of GTE4 were negative, indicating that GTE4 is not forming homodimers. This assay does obviously not exclude the possibility that GTE4 forms heterodimers with other BET proteins.

**The gte4 mutant presents defects in aerial organ size and shape**

To functionally analyse GTE4 we searched the Salk T-DNA collection (Alonso et al., 2003) for GTE4 insertion mutants. In this collection, we identified a line that has a T-DNA insertion 200 bp downstream the bromodomain (Fig. 2A). Homozygous mutant lines were identified by PCR and confirmed by Southern blot analysis (not shown). Expression analysis by RT-PCR using RNA extracted from the homozygous gte4 mutant showed that the T-DNA insertion caused complete silencing of the GTE4 gene (Supplemental Fig. S2).
Initial phenotypic analyses of the gte4 mutant showed that these plants were significantly reduced in size at all stages of plant development (Fig. 2B and C). Comparing leaves of wild-type (wt) and gte4 mutant plants evidenced that those of the mutant were not only smaller but also had a slightly serrated shape (Fig. 2D). The production of rosette leaves was delayed in the gte4 mutant, with a significant (P<0.01) reduction in leaf number up to day 14. At day 20, the mean number of rosette leaves had become the same (6.6 ±0.3 for wt and 6.6 ±0.4 for gte4), however differences in leaf morphology remained. The leaves of gte4 plants, e.g., the 6th leaf, were significantly (P<0.05) smaller than those of wt (Table I). Histological analysis carried out on the 6th leaf showed a higher variability in the cellular dimensions of the mutant leaf in comparison with the wt leaf (as shown by SEs in Table I), however the mean areas of the subepidermal (Table I) and epidermal (not shown) cells were not statistically different between the genotypes. Moreover, there was no significant change in the mean area of the intercellular spaces (data not shown). However in the gte4 mutant the number of cells in the subepidermis was about half in respect to wt (Table I). This suggests that in the gte4 mutant there is a general reduction in leaf cell number. During later developmental stages the leaf phenotype of gte4 plants was not rescued, in fact, at day 35 the leaves remained significantly (P<0.05) smaller than wt leaves (Table I).

The floral transition was not affected in the gte4 mutant and occurred under long-day conditions at the same time (day 16) as in wt plants. However, in gte4 plants the height of the inflorescence stem remained significantly (P<0.01) reduced up to end of the life cycle [i.e. 23.3 (±0.8) versus 27.9 (±1.3) cm for wt] (Fig. 2B). Stem thickness was also significantly (P<0.01) reduced, which was due to a reduction in the mean area of the cortex and stelar regions (Fig. 2E; Supplemental Table SI). These differences were due to a reduced cell number, as exemplified for the cortex in Supplemental Table SI.

Analysis of gte4 flowers revealed no differences in floral organ size. The only difference detected in floral structures was a reduction in stamen number. In wt flowers we always observed 6 stamens whereas in the gte4 mutant this number was reduced to 4.7±0.2 (Fig. 2F). Some delay in silique growth also occurred, however at the end of the reproductive phase neither the length of the siliques nor the number of seeds per silique changed in comparison to wt (data not shown).

The gte4 mutant affects root development

Root development was clearly affected in gte4 mutant plants. Significant differences in primary root length were already observed at day 4 (Fig. 3A and B). The gte4 mutant roots were about three-times shorter than the wt ones (Table II). The mutant hairy region was about 25% of the length compared to wt whereas the elongation region was about 50% reduced in length. The cell area in both the elongation region and in the hairy region was not significantly different till day 4, which
shows that there is a clear reduction in cell number in both regions of the mutant (Table II). At day 9, the reduction in cell numbers was enhanced in the mutant (Table II). The roots of the mutant continued to be shorter than wt after day 9, although the difference in length was progressively reduced (data not shown).

At 4 days after germination the primary root apical region was analysed in detail, starting from the basal walls of the quiescent center (QC) cells up the distal border of the elongation region (Fig. 3C and D). The length and area of this region were significantly reduced in mutant roots in comparison with the wt ones (Table III). The cell size in the gte4 mutant was instead more than twice that of wt cells, resulting into a highly reduced cell number in gte4 apices (Table III). The length and area of the apex did not change during the following days in both genotypes, as shown for day 9 in Table III. The only difference was that in wt primary roots the mean cell size reached a value comparable with those measured in the gte4 mutant. However, since the region area in wt remained more than twice that in mutant roots, the cell number in the gte4 mutant continued to be highly reduced (Table III).

Also the development of the lateral roots, and their contribution to the root apparatus was different in gte4 plants, both considering the timing of their macroscopic protrusion from the primary root and their number. In fact at day 9, in comparison with wt roots lateral root formation was still poor on gte4 plants. At day 20, the differences in lateral root formation continued to be observed, with a significant lower number of lateral roots (P<0.01) in the mutant [i.e., 1.6 (±0.6) compared to 6.9 (±1.3) in wt] and a reduced density (data not shown).

At day 9 the root apex of lateral roots was histologically examined. Besides the difference in cell number and density when compared to wt roots (see above), the lateral roots seemed to develop slower in the mutant. In gte4 plants the lateral roots where still primordia whereas in wt roots they were already in a much more advanced stage of development (Fig. 3E and F). The apical dome of the emerging lateral roots exhibited the same shape in the two genotypes (Fig. 3G and H) and as a consequence its area was similar (Table III). The mean cell area in the mutant was instead higher (P<0.05), resulting into a reduced (P<0.05) cell number in comparison with wt (Table III).

**Loss of GTE4 function affects seed development and germination**

To investigate whether the gte4 mutant already demonstrated developmental defects at the embryonic stage we analysed the formation of the embryo and the suspensor during seed development. This analysis showed that no visible changes occurred in the mutant embryo from octant to heart stage (Fig. 4A-D). However, the area of the suspensor cells was significantly (P<0.05) increased in the mutant relative to the wt (i.e., 148.9±15 μm² and 109.2±9.3 μm², respectively). This resulted into a more elongated suspensor in gte4 at the heart stage (Fig. 4C,D).
Moreover, at the cotyledonary stage about half of the \textit{gte4} embryos showed anomalous root pole development (Fig. 4E-H), with irregular shaped QC cells and irregularly dividing columella initials (Fig. 4G and H).

The root pole of the mature embryo was also analysed after 5 hours imbibition on filter paper (Fig. 4, I and J). Differences in the shape and size of the most apical region (50 µm in length, starting from the basal walls of the QC) were observed (i.e., 2305.1±64.8 µm² and 2692.1±71.5 µm² for \textit{gte4} and wt, respectively, P<0.01). Since dimensions of the embryonic cells did not change significantly between the genotypes (i.e., 66±1.9 µm² in \textit{gte4}, and 60.1±2.3 µm² in wt), the cell number in this region resulted significantly (P<0.01) reduced in the mutant embryos in comparison to those of wt (i.e., 35±0.6 and 45.1±1.3, respectively).

To test whether seed germination was affected we plated wt and \textit{gte4} mutant seeds on filter paper for 7 days. This analysis showed that after two days 75% of the wt seeds were germinated whereas only 8% of the mutant seeds germinated. The delay in germination continued up to day 4 (67% of \textit{gte4} germinating seeds with respect to 98% of wt seeds, Table IV). Finally after one week both wt and mutant seeds had all germinated indicating that there is only a delay in germination not a defect that completely abolishes germination of the mutant seeds.

**The \textit{gte4} mutant shows defects in the quiescent centre**

Histological analysis of the \textit{gte4} mutant showed that QC cells were always present in the primary root apex (Fig. 3C and D). QC cells were normally defined during embryogenesis, however, they became progressively smaller and, starting from day 4, confined with irregularly dividing cells, procambial ones in particular (Fig. 3C and D, insets). During the formation of lateral roots in the \textit{gte4} mutant, QC cells were normally produced, and exhibited a normal shape at the early emergence stage of the primordium (Fig. 3G and H). At the following expansion phase (Malamy and Benfey, 1997) the QC cells appeared to be more expanded than in wt. The same was observed for some columella initials that surround the QC (Fig. 3, I and J). Subsequently, in the protruded lateral roots QC cells became highly reduced in size (Fig. 3, K and L), whereas cells surrounding the QC were irregular in size (Fig. 3L inset) and columella initials were expanded (Fig. 3, K and L, inset), resulting into an anomalous morphology of the apical meristem.

These observations suggest that QC cells are not functioning correctly in mature apices, and this might result in a lack of coordination in division activity of the surrounding initials, affecting macroscopic organ development.

To investigate whether the loss of GTE4 function causes QC defects we crossed the \textit{gte4} mutant with three QC marker lines: two QC-expressed promoter trap GUS lines, QC25 and QC46 (Sabatini et al., 2003), and the AGL42:GFP line (Nawy et al., 2005). This analysis showed that in the \textit{gte4}
mutant the QC25 and QC46 markers are normally expressed (Fig. 5C-F). However, in the gte4 mutant containing the AGL42:GFP marker no GFP signal could be detected in all stages of root development following QC definition (Fig. 5A-B), thus indicating a partial, but not transient loss of QC identity. This might explain the observed defects in the root meristem morphology.

**Loss of GTE4 function affects cell proliferation in the root meristem and promotes endoreduplication in both roots and shoots**

The onset of cell proliferation during seed germination was investigated by applying the bromodeoxyuridine (BrdU) incorporation/immunodetection method. The BrdU labelling allows the detection of all the cells that enter S-phase at least once during the period in which BrdU is present in the medium. Wild-type and gte4 mutant seeds were imbibed and grown for 96 hours in the presence of BrdU. No labelled nuclei were detected up to 12 hours in both wt and mutant roots (Table IV). At 24 hours, a low percentage of proliferating cells (4%) were only revealed in wt roots. Such percentage increased in the next hours reaching a mean value of 89% after 48 h. At 96 hours nearly all wt root cells were BrdU positive. Similar kinetics for cell cycle activation was observed in gte4 mutant root as seeds germinated. However, most seeds had not yet germinated after 48 hours. In these seeds no cycling cells were detected (Table IV, supplemental Fig. S3), suggesting that the delay in root emergence might be related to the delay in cell cycle activation.

Additional analyses to investigate for anomalies in cell cycle regulation were performed on roots and shoots applying the BrdU pulse/chase method along with the determination of mitotic index and flow cytometry (FCM) (Galbraith et al., 1991). Three-day-old wt and mutant seedlings, incubated with BrdU during the last two hours of growth, showed no significant difference in the percentage of root meristem cells which were running either through S-phase or M-phase (Table V). This indicated that the loss of GTE4 function did not affect the cell cycle progression in the root meristem of 3-day-old plants. Nevertheless, the analysis of the 4- and 5-day-old seedlings showed that the proportion of cells that underwent mitosis was significantly reduced (P<0.01) in mutant root meristem, whereas the percentage of S-phase traversing cells was similar to that observed in wt (Table V). This suggested that in the mutant a substantial number of cells exit from the mitotic cell cycle earlier than the corresponding wt cells and switched from mitosis to endoreduplication. Accordingly, FCM analysis of 4- 5-day old roots revealed a statistically (P<0.05) higher percentage of 8C polyploid cells in the gte4 mutant, whereas no difference occurred in 3-day-old roots in comparison with wt (Table VI A, B and C). The enhanced endoreduplication in gte4 roots was confirmed by the analysis of fully differentiated plant organs (e.g., differentiated portions of roots from 10-days- and 30-days-old seedlings) in which the percentage of polyploids was significantly higher (P<0.01, Table VI D and E). Similar results were obtained for the leaves. Like in 3-days-old
roots, in the first formed leaf primordia cell cycle progression was not affected (Table VI F), whereas enhanced endoduplication occurred in the fully differentiated leaves and cotyledons like in roots (P<0.01 Table VI G and H).

Based on these results GTE4 plays a role in the control of cell proliferation and it can be supposed to be involved in the retinoblastoma (RB)-E2F pathway, which is one of the most important pathways involved in the control and coupling of cell division and cell differentiation in both animals and plants (van den Heuvel and Dyson, 2008). To test this hypothesis we analysed by quantitative RT-PCR the expression of a few genes that are involved in the E2F pathway in 4-days-old plantlets.

We tested E2Fc, E2Fe/DEL1, CDC6 and CCS52A2. EF2c was selected because it was shown to function at two stages during the cell cycle: at G0 (cell cycle enter), during which E2Fc actively represses E2F target genes, and later in the cell cycle, likely in G2/M, when E2Fc might participate in the decision to progress through mitosis or to switch to the endocycle and differentiation programs (del Pozo et al., 2006). E2Fe/DEL1 expression was tested because it is another important cell cycle regulator. It is an inhibitor of the endocycle and preserves the mitotic state of proliferating cells by suppressing transcription of genes that are required for cells to enter the DNA endoreduplication cycle (Vlieghe et al., 2005). Finally, CDC6 and CCS52A2 were selected among the E2F target genes because CDC6 is critical for DNA replication and its mRNA level is reduced by the overexpression of the non degradable form of the E2Fc protein, whereas CCS52A2 is critical for the onset of endoreduplication and is a direct E2Fe/DEL1 target (Lammens et al., 2008; del Pozo et al., 2006; Castellano et al., 2004).

From this analysis emerges that, except for E2Fe/DEL1 which expression seems to be not significantly changed, E2Fc and CCS52A2 expression is significantly upregulated and CDC6 is down-regulated (Fig. 6). These changes in expression are in agreement with a possible involvement of GTE4 in the RB/E2F pathway for the control of cell proliferation.

Rescue of the gte4 mutant phenotype by complementation

To provide proof that the observed phenotypes of the gte4 mutant are due to the loss of GTE4 activity a complementation experiment was performed. Plants homozygous for the gte4 allele were transformed using a binary vector carrying a genomic fragment that included sequences 1.6 kb upstream of the translation start site, the entire coding region and 947 bp down stream of the stop codon. Transformants were identified by kanamycin selection and the presence of the complementation construct was confirmed by PCR. We obtained 12 transformants (homozygous for
the *gte4* allele) of which 7 had a normal wild-type phenotype (not shown). This confirms that the introduced construct complements the *gte4* phenotypes.

**DISCUSSION**

GTE4 is a member of the BET protein family which are known to contain a bromodomain capable of interacting with acetylated lysines usually found in histones and an ET-domain which is a protein-protein interaction motif (Denis et al., 2006). These sequence characteristics make BET proteins key players in the modulation of gene expression by epigenetic mechanisms (Yang et al., 2008, Florence and Faller, 2001). Epigenetic mechanisms regulate genome reprogramming during early embryogenesis and gametogenesis, cell differentiation and maintenance of a committed lineage (Delcuve et al., 2009).

BET proteins have been studied in a variety of animal organisms where they are mainly implicated in cell cycle progression by transmitting epigenetic memory through mitosis (Yang et al., 2008; Florence and Faller, 2001). In plants until now, only two members of the *Arabidopsis thaliana* BET gene family have been characterized, i.e., *IMB1* and *GTE6*, with a role in the promotion of seed germination and leaf shape, respectively (Duque and Chua, 2003, Chua et al., 2005).

Here we report the characterization of *GTE4*, which belongs to a different phylogenetic group of the Arabidopsis BET family of which no member was studied yet. Phenotypic characterisation of the *gte4* mutant shows a variety of developmental defects in roots, leaves and flowers which might be caused by a defective cell cycle regulation in meristematic cells leading to a significant reduction in cell number and a significant increase in ploidy level in most organs. Interestingly in this mutant the functionality of the root quiescent center (QC) also seems to be partially lost.

In the Arabidopsis root meristem, the initial cells produce mitotically active derivative cells which further differentiate giving rise to all cell types of the mature root. The initial cells surround a small group of mitotically less active cells that is called the QC. The QC cells and the initials form the stem cell niche (Sabatini et al., 2003). Loss of QC identity causes the loss of the stem cell niche and prevents the root from growing (Aida et al., 2004, Tucker and Laux, 2007).

The *gte4* mutation affects the root stem cell niche and derivative cells from the very first stages of development. This is demonstrated by the fact that the embryo root apex shows after 5 hours of imbibition a reduced number of cells, and the morphology of the QC and surrounding cells is abnormal in a large proportion of embryos at the cotyledonary stage.

The aberrant morphology of the *gte4* QC and surrounding cells is likely correlated to the partially loss of QC identity, shown by the lack of the QC-specific pAGL42::GFP reporter expression in these mutant plants and the presence of QC25 and QC46 promoter trap expression. Partial loss of
QC identity has also been observed in the *scarecrow* (*scr*) mutant. In this mutant the QC184 marker maintains its expression in the QC, whereas the QC25 and QC46 lose their expression (Sabatini et al., 2003). Interestingly, cells in the *scr* QC region are aberrant in shape and roots ultimately cease growth (Scheres et al. 1995; DiLaurenzio et al. 1996). *Gte4* roots are less affected than *scr* roots, since root growth is not completely abolished. However, *gte4* QC cells, and surrounding ones, have an aberrant morphology and the root meristem is disorganized, showing altered pattern formation.

The analysis of the cell cycle in developing seedlings revealed a premature arrest of the cycling cells which switch from mitosis to endoreduplication. No anomalies in cell cycle progression through the cell cycle phases were instead detected. These results indicate that *gte4* meristematic cells can normally proliferate, but they are incapable to balance the number of differentiated cells with the maintenance of an adequate pool of self-renewing cells. This failure to properly coordinate differentiation with the permanent exit from the cell cycle should explain the phenotypic characteristics of *gte4* mutant, i.e. small organs composed of a reduced number of cells.

An additional feature of *gte4* mutant plants is that the differentiated organs are composed of a statistically higher amount of polyploid cells. As mentioned above, cell cycle analysis showed that the premature arrest of the mitotic cell cycle is coupled with the onset of endoreduplication which leads to an increase in ploidy level. The link between mitotic cell cycle and endoreduplication has been widely investigated, and these data suggest that endoreduplication is achieved by a modification of the mitotic cell cycle. For instance Arabidopsis plants with a reduced CDKB1;1 activity prematurely exit the mitotic cell cycle and have elevated ploidy levels (Boudolf et al. 2004). Similarly the Arabidopsis *SIAMESE* (*SIM*) gene is required to suppress mitosis as part of the switch to endoreduplication in trichomes (Churchman et al. 2006). Thus, *GTE4* seems to be involved in the maintenance of mitotic cell cycle, and the endoreduplication observed in *gte4* mutant seems directly linked to the premature cell cycle exit, and might be positively related to the cell size increase observed in some organs. An increase in cell size in response to the inhibition of cell division, and induction of endopolyploidy, has also been observed previously, and can be attributed to an uncoupling of cell division and cell expansion (De Veylder et al. 2001; Sugimoto-Shirasu and Roberts, 2003). This hypothesis has already been formulated for other mutants with a reduced cell number, such as the Arabidopsis *struwwelpeter* (*swp*) mutant (Autran et al., 2002). Similarly to *GTE4*, *SWP* plays a role in pattern formation in the meristem and is important for defining the duration of cell proliferation.

Finally, the analysis on the onset of cell cycle in *gte4* germinating seeds showed a delay in cell cycle reactivation in comparison to wt. This delay might explain the slower protrusion of the embryonic root from the seed coat registered for the mutant. Moreover a delay in cell cycle activation in the pericycle founder cells could also explain the drastic delay in lateral root formation.
that we observed in the *gte4* mutant. Many studies both in animals and plants suggest a tight relationship among the onset of the cell cycle, the maintenance of cell proliferation and differentiation. For instance Dewitte et al. (2003) demonstrated that Arabidopsis CYCD3 promotes the mitotic cell cycle and affects differentiation by inhibiting mitotic exit and/or endocycles, either independently or through its regulation of Rb function. In mammalian development, in particular, the Rb-E2F pathway is of central importance and represents the link between the activation of cell proliferation and cell cycle exit, leading to terminal differentiation (Kirshenbaum, 2001).

Thus *GTE4* seems to regulate not only the maintenance of meristem cell proliferation but also the onset of cell cycle. The pathway(s) that is affected by the *gte4* mutation is currently not defined, although on the basis of literature it is possible to give some hypotheses. It has been shown that the re-entrance in the cell cycle is preceded by a change in chromatin condensation. Heterochromatin starts to unpack and new euchromatic regions, compatible with transcriptional activity, are formed (Zhao et al., 2001; Williams et al., 2003). These epigenetic changes might be mediated by GTE4 through its ability, as BET protein, to interact with acetylated histone tails. We can therefore hypothesize that GTE4 may play a role in translating the histone acetylation marks into cell cycle gene activity allowing the entrance into the cell cycle. GTE4 is also necessary to maintain cell proliferation in the root meristem, since its role does not seem to be limited to re-entry in cell cycle. E2F transcription factors have also shown to regulate these two processes. E2F are responsible for stem cell maintenance in the root, regulating the transition from cell proliferation to differentiation and are involved in the re-activation of cell proliferation in the QC (Wildwater et al., 2005; Lammens et al., 2008). In addition, when a cell is re-entering into the cell cycle the chromatin around E2F target genes becomes decondensed allowing E2F to regulate target gene transcription (Williams et al., 2003). Moreover, in mammals, the decision of cells to continue or stop dividing depends largely on the activity of the E2F transcription factors (van den Heuvel and Dyson 2008). In Arabidopsis, six E2F (E2Fa-f) and two DP (a and b) proteins were identified, and some of them were demonstrated to be key regulators of cell proliferation and endoreduplication (De Veylder et al., 2002). For instance, E2Fc-DPb restricts cell division and is one of the components in the coordination between cell proliferation and endoreduplication during *Arabidopsis* development (Del Pozo et al., 2006). In agreement with an involvement of GTE4 in the RB-E2F pathway, 4-day-old *gte4* mutant plantlets showed changes in the expression of *E2Fc*, *CDC6* and *CCS52A2* genes. Specifically, *E2Fc* was significantly up-regulated and its target gene *CDC6* down-regulated. Interestingly, like plants overexpressing E2Fc/DPb, *gte4* mutant plants are characterized by a reduced number of mitotic cells and an increase DNA content. Moreover, also the expression of the APC/C activator gene *CCS52A2* was significantly enhanced in 4-day-old *gte4* plantlets, suggesting
a transcription deregulation of genes important for a correct cell proliferation/endoreduplication and related to the RB/E2F pathway.

Due to these analogies we can speculate that GTE4 might be involved in E2F related pathways controlling gene transcription. This hypothesis is also supported by the fact that the BRD2 (RING3) BET bromodomain protein in animals binds to E2F and together they regulate gene transcription and cell cycle activity (Denis et al., 2000).

Although our analyses suggest a link between GTE4 and E2F related pathways some caution has to be taken since the number of genes that we analysed is limited and we do not know if the observed changes in expression levels are physiologically relevant and if the effects on the expression of these genes are direct or indirect. Future studies directed to identify target genes that are under the control of GTE4 will be needed to draw a clearer picture of the regulatory pathways that are controlled by this plant BET protein and to get a better understanding of its role in epigenetic control of plant development.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The Arabidopsis thaliana gte4 mutant (ecotype Columbia) was obtained from the SALK collection SALK_113292 Code N613292. Seeds were vernalized for 2 days at 4°C under continuous darkness, sterilized for 10 min in 10% sodium hypochlorite and then rinsed with three changes of sterile distilled water and sown in Petri plates on half-strength MS (Murashige and Skoog) supplemented with 0.55 mM myo-inositol, 0.3 μM thiamine-HCl, 2% (w/v) glucose, and 0.8% agar (w/v, Sigma). Seeds were incubated under long day conditions (16/8 h light/dark) at a fluence rate of 150 μEm⁻²s⁻¹ and 22±2°C. The plates used for the root apparatus analyses were oriented vertically to ease the observation and removal of roots (Malamy and Benfey, 1997). For observation of germination, vernalized seeds were sterilized and put on filter paper for 7 days, whereas for the observation of aerial organs, the seeds were imbibited on filter paper for 2 days at 4°C and sown on commercial soil (Universal Soil, Manna, Italy). The same growth chamber was used as described above.

QC46 and QC25 promoter trap GUS lines were kindly provided by S. Sabatini (Sabatini et al., 2003), and the AGL42:GFP line by P. Benfey (Nawy et al., 2005).
Phylogenetic analysis

We produced a rooted tree for these Arabidopsis BET proteins using the bromodomain and the ET domain. The protein sequences have been analyzed using the program ClustalX for protein sequence alignments (Jeanmougin et al., 1998). The tree was obtained using Phylip (Retief, 2000).

RT-PCR

RT-PCR reactions were performed on cDNA obtained as described previously (Lago et al., 2004) using the following primers specific for GTE4. Atp 388 (5’GATCAGCTTAACGAGTAGTCAGAG) and Atp 389 (5’CGTCTACTGGAGCATTGAACAC). The PCR reaction was performed for 30 cycles using an annealing temperature of 54°C.

Quantitative Real-time RT-PCR

RNA was extracted using Quiagen RNasy Plant Mini Kit (cat. No. 74904), followed by DNAse treatment performed like in (Lago et al., 2004). Invitrogen SuperscriptII has been used to reverse transcribe the RNA following the manufacturer instructions. Real time PCR has been performed with a Biorad IQ5 machine using IQ Biorad SBR Green Supermix (cat. no. 170-8882) and the primers below. Primer annealing was set at 59°C for 40 cycles and ACTIN has been used as reference gene. Two separate Real time PCRs have been performed with three replicas for each sample. The melting and standard curve has been determined for each Real time PCR.

Real time PCR primers:

ACTIN2_f GCTCCTCTTTAACCAACCCAGGC
ACTIN2_r ACACCATTACCAGGGTCCAGCC
CCS52A2_f ACTCGTACCAGCTTCTCTGAC
CCS52A2_r CTCCCTGCTCTGAGATTTGC
CDC6_f GCCGGACACTTAGTTCTCC
CDC6_r GAAAACCTCCGACCCGAAATC
E2Fc_f GAGTCTCCTCCACGATTTGC
E2FC_r TCACCATTCCCCCTCAG
DEL1_f GTCCCAAGAAACGCTACAGAG
DEL1_r AGTGCGCTGGTCAAAAGGTC
Mutant analysis
The position of the T-DNA insertion in the GTE4 gene was identified by PCR and subsequent sequencing using primers Atp 388 and Atp58 (5’TGGTTCACGTAGTGGGCCATCG) T-DNA specific primer.

Histological analysis
The histological analysis was carried out on leaves, stems, roots, siliques and seeds.
Leaves were fixed in 70% ethanol, dehydrated, embedded in Technovit 7100 (Heraeus Kulzer, Germany), sectioned at 4 µm with an automatic microtome (Microm HM 350 SV), stained with 0.05% (w/v) toluidine blue, and examined under a DAS Leica DMRB microscope (Leica, Heerbrugg, Switzerland). Alternatively, the leaves were treated with chloral hydrate solution (chloral hydrate:distilled water:glycerol, 8:1:2 w/v/v) for the observations with Nomarski optics applied to the same microscope.
To evaluate stem diameter, cortex area, stelar area, cortex cell area and number, the median stem internodes of 55 days-old wild-type and gte4 plants were embedded in 5% (w/v) agar, sectioned at 30 µm with a vibratome (Vibratome Series 1000), and observed by Nomarski optics. Embryos were analysed in siliques and mature seeds. The siliques were fixed, dehydrated, embedded, sectioned and stained as previously described, the seeds were imbibited on filter paper for 5h and treated for the observation by Nomarski optics.
Primary and lateral root morphology, and QC and surrounding cells were analysed by Nomarski optics. In the primary root, the length of the hairy region was measured from the most proximal (i.e. towards the shoot apex) to the most distal (i.e. towards the root apex) trichoblast, that of the elongation region from the most distal tricoblast to the most proximal cortical cell showing elongation, and that of the apical region from this latter cell to the basal walls of the QC cells. To calculate the area of the apical region of the primary root, it was assimilated to an isosceles triangle, whose height was the length of the region, measured as described above, and the base was the width of the region, measured, including the root protoderm, at the distal border of the elongation region. To calculate the area of the apical region in the lateral root primordium, it was assimilated to an isosceles triangle, whose height was 50 µm, measured proximally from the basal walls of QC cells, and the base was the width of the primordium at the proximal end of the height.

Histochemical Analysis of GUS Activity and GFP Analysis
Gte4 mutant plants were crossed with the QC25, QC46 and pAGL42:GFP reporter lines and from the F2 population gte4 homozygous plants containing the reporter constructs were selected and used to analyze the QC through GUS activity and monitoring GFP fluorescence by a Leica DMRB
microscope equipped with a double wavelength filter set (BP 490/20 and BP 575/30) dichroic filters RKPs 505 and 600, and emission filters BPs 525/20 and 635/40. All the histological images were acquired with a DC500 video camera applied to the DMRB microscope and then analysed with a personal computer (Opti-Xex GX 240 MT) using the Leica IM1000 image analysis software (Leica).

**Statistical Analysis**

Differences between percentages were evaluated using \( \chi^2 \) test, and differences between the means by the Student's \( t \)-test, using GraphPad InStat3 software.

**Complementation of the gte4 Mutant**

The BAC clone F9P14 was used to obtain the \textit{GTE4} genomic region for the complementation experiment. The BAC clone was digested with Sall and KpnI and a band of 3900 bp was isolated containing a part of the At1G06230 locus and inserted into P-Cambia 1300. To add the 3’ UTR region we performed a PCR with AtP597 (5’AACCATATGCTACCAATGTCTG) and AtP598 (5’GGGAGCTCAGTGGCTACCTTGCTTCCACAC). The amplified fragment was cloned into the pGem-T-Easy vector (Promega). Subsequently, this fragment was inserted as a KpnI-Sacl fragment into the binary vector pCAMBIA 1300 already containing part of the At1G06230 locus. This binary vector was used to transform \textit{Agrobacterium tumefaciens} C58C1/pMP90 (Koncz et al., 1984). Arabidopsis plants were transformed using the floral dip method described by Clough and Bent, (1998).

**Cell Cycle Analysis**

\textit{Flow cytometric analyses}

Nuclear suspensions were obtained from Arabidopsis plants at different development stages following the protocol of Galbraith et al. (1983). Chicken erythrocytes were added as reference internal standard to each sample. The mixed nuclei were stained with the DNA binding fluorochrome DAPI at a final concentration of 5.5 \( \mu \)M. The fluorescence intensity of the nuclei was measured with an arc lamp-based flow cytometer (Bryte-HS; Bio-Rad, Hercules, CA). Four independent experiments were carried out. In each experiment 450 pooled plantlets were subjected to flow cytometric analysis according to organ type: at least 5 pools of about 100 plantlet leaf primordia, 5 pools of 10 cotyledons and at least 5 pools of about 50 whole roots were analysed in 3, 4, 5 and 10 day-old plantlets. In 3, 4, 5 and 10 day-old wt plantlets the analysed roots were 2.5±0.2, 3.1±0.2, 5.7±0.9 and 17±0.9 mm long, respectively. Smaller lengths (0.73±0.1, 0.97±0.2, 1.5±0.5, 7.4±1.0) were recorded for the analysed \textit{gte4} roots at the same growth stages. About 20 plants were
instead individually analysed to determine ploidy in mature leaves and roots excised from 30 days-old plants. All the rosette leaves (about 9±0.3 leaves for both wt and gte4) and the whole root were analysed for each plant. The significant differences between gte4 and wt mean percentages were statistically analysed by the Statgraphics plus program for Windows (version 4.0, Manugistic, Maryland USA): ANOVA and Dunnet tests were applied when normality and homogeneity of variance were satisfied; data which did not conform to the assumptions were alternatively transformed into logarithms or were analysed by Kruskal-Wallis non-parametric procedures.

**Bromodeoxyuridine incorporation and detection**

In order to study the kinetic of cell cycle reactivation in root meristem during germination, plantlets were germinated from surface-sterilized seeds at 25 °C on filter paper imbibed with 10 µM BrdU solution (Sigma- Aldrich , San Louis, Mo, USA). Root tips were excised after 72h from the start of imbibition and were fixed in 4% (w/v) paraformaldehyde (Polysciences, Warrington, PA, U.S.A; 10% solution, methanol free) in Tris buffer (10 mM tris(hydroxy-methyl) aminomethane, 10 mM NaEDTA and 100 mM NaCl, pH 7.4) for 16 h at 4 °C. Root tips were also collected and fixed from 3-, 4-, and 5-days-old plantlets grown on filter paper imbibed with distilled water and pulsed with 30 µM BrdU only during the last two hours of growth, to detect the percentage of meristematic cycling cells. After fixation, all the samples were embedded in London Resin Gold (L.R. Gold; Polysciences Europe, Eppelheim, Federal Republic of Germany). Sections were obtained with a Reichert Jung Ultracut E microtome (Reichert, Vienna, Austria) and were collected on poly-L-lysine-coated slides. Selected sections were used for immunochemical detection of BrdU according to standard protocols. A negative control sample, without BrdU, but with the rabbit primary antibody, was also included in the experiment. Slides were examined with a Zeiss Axioplan microscope (Zeiss, Oberkochen, Federal Republic of Germany) equipped with a video camera (Media Cybernetics, Silver Spring, Md., U.S.A.). The acquired digital images were analyzed by the Image-Pro Plus program (Media Cybernetics). The experiments were all repeated at least 3 times. In each experiment at least 20 randomly chosen roots were analysed per genotype and developmental stage. The figures show the results of one representative experiment. The tables report the means (±SD) of the data collected in all the experiments and their statistical analysis.
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Table 1. Microscopic analysis of rosette leaves of 20 and 35 days old gte4 and wild-type plants

|          | wild-type       | gte4            |
|----------|-----------------|-----------------|
|          | leaf area (mm²) | 31.4 ± 3.4b,d   | 17.7 ± 3.9c     |
| d 20     | cell area (μm²) | 516.5 ± 31.6c   | 554.2 ± 130c    |
| cell number | 60947.5 ± 6044.6a | 34375.1 ± 3168.1 |
| d 35     | leaf area (mm²) | 50 ± 4b         | 37.4 ± 1.1      |
| cell area (μm²) | 1031.8 ± 118     | 1017± 129.3     |
| cell number | 50186.9 ± 4218.3b | 36792.6 ± 3957.4 |

The mean area (± SE) of the 6th rosette leaf, the mean area of the subepidermal cells (± SE), and the mean number of subepidermal cells are shown at days 20 and 35 (70 cells randomly chosen in 10 specimens per genotype).

a Values of wt differ statistically from the gte4 mutant (P< 0.01); b values of wt differ statistically from the gte4 mutant (P< 0.05); c values of day 20 is statistically different with respect to the corresponding values of day 35 within the same genotype (P<0.01); d values of day 20 is statistically different with respect to the corresponding values of day 35 within the same genotype (P<0.05). Values followed by no letter or by the same letter are not significantly different.
Table II. Histological analysis of the hairy and elongation regions in the primary root of 4 and 9 days old gte4 and wild-type seedlings

|       | wild-type |                       | gte4          |                       |
|-------|-----------|------------------------|---------------|------------------------|
|       | Hairy region | Elongation region | Hairy region | Elongation region |
| d 4   | length (μm) | 2342.3 ± 210.5 a | 565 ± 45.5 a | 594.8 ± 82.9 | 308.8 ± 39.2 |
|       | cell area (μm²) | 1571.4 ± 151 | 587.6 ± 122.1 | 1861.5 ± 131.5 | 369.9 ± 95.8 |
|       | cell n./region | 202.4 ± 19 a | 118.7 ± 15.3 b | 66.6 ± 6.3 | 95.3 ± 20.6 |
| d 9   | length (μm) | 15406.3 ± 1181.4 a | 509.7 ± 30 a | 6419.5 ± 1023.9 | 342.5 ± 23.4 |
|       | cell area (μm²) | 1467.8 ± 66.9 | 384.9 ± 11.7 | 1654.7 ± 258 | 577.8 ± 113.2 |
|       | cell n./region | 1265.4 ± 109.9 a | 155.3 ± 12 a | 479.6 ± 145.7 | 68.7 ± 15.5 |

Mean length, mean cell area, and mean cell number (± SE) of cortical cells in the hairy region and in the elongation region are shown at days 4 and 9 (100 cells randomly chosen in 10 specimens per genotype). a Values statistically different (P< 0.01) with respect to the corresponding region in gte4 genotype at the same day; b Values statistically different (P< 0.05) with respect to the corresponding region in the gte4 mutant at the same day. Values followed by no letter are not significantly different between genotypes in the same region at same day.
### Table III. Mean length and area of the root apex of primary and lateral roots in 4 and 9 days old gte4 and wild-type seedlings

|                  | Primary roots |               |               |
|------------------|---------------|---------------|---------------|
|                  | wild-type     | gte4          |               |
| length (μm)      | 163.2 ± 4.0   | 88.6 ± 9.3    |               |
| region area (μm²)| 9671.9 ± 717.6 | 4137.6 ± 797.2 |               |
| cell area (μm²)  | 46.1 ± 2.2    | 93.9 ± 15.7   |               |
| cell n./region   | 216.1 ± 16.2  | 42.6 ± 8.9    |               |

|                  | Lateral roots |               |               |
|------------------|---------------|---------------|---------------|
|                  |               |               |               |
| d 4              |               |               |               |
| length (μm)      | 188.8 ± 13    | 104.4 ± 9.8   |               |
| region area (μm²)| 11052.4 ± 486.1 | 4851.4 ± 563.5 |               |
| cell area (μm²)  | 95.1 ± 10     | 96.1 ± 10.1   |               |
| cell n./region   | 121.5 ± 12.7  | 51.0 ± 5.5    |               |

The mean cell area and cell number of the cortical derivative cells (± SE) are shown (30 cells randomly chosen in 10 specimens per genotype and day for the primary root, and 10 cells randomly chosen in 10 specimens per genotype for the lateral root).

*Values statistically different (P < 0.01) with respect to the gte4 mutant. bValues statistically different (P < 0.05) with respect to the gte4 mutant. Comparative values between wt and gte4 followed by no letter are not significantly different.
### Table IV Germination and Cell Cycle Analysis

**BrdU continuous labelling**

| Time from start of seed imbibition (h) | Percentage of germination | BrdU positive nuclei (%) |
|---------------------------------------|---------------------------|---------------------------|
|                                       | Wild-type | Mutant | Wild-type | Mutant |
|                                       |           |         | Germinated | Non-germinated |
| 12                                    | 0         | 0       | 0          | 0       |
| 24                                    | 0         | 0       | 4 ± 1\(^1\) | 0       |
| 48                                    | 75 ± 2    | 8 ± 5\(^1\) | 89 ± 12 | 87 ± 9 |
| 72                                    | 95 ± 4    | 55 ± 7\(^1\) | 98 ± 6  | 98 ± 11 |
| 96                                    | 98 ± 7    | 67 ± 9\(^1\) | 99 ± 12 | 98 ± 13 |

Values are the means (±SD) of the percentages obtained in 3 independent experiments. In each experiment the percentage of BrdU positive nuclei was determined in 20 randomly chosen root tips per genotype and developmental stage.

\(^1\) These values are statistically different (P<0.01)
### Table V Cell Cycle Analysis

**BrdU pulse and chase labelling and mitotic index determination**

| Time from start of seed imbibition (h) | Percentage of S-phase traversing cells (BrdU positive cells) | Percentage of M-phase traversing cells (Mitotic Index) |
|---------------------------------------|-------------------------------------------------------------|------------------------------------------------------|
|                                       | Wild-type | Mutant | Wild-type | Mutant |
| 72                                    | 18.1 ± 1.1 | 16.7 ± 0.9 | 4.8 ± 0.2 | 4.5 ± 0.4 |
| 96                                    | 15.3 ± 0.7 | 16.0 ± 1.1 | 4.2 ± 0.1 | 2.1 ± 0.3$^1$ |
| 120                                   | 14.2 ± 1.3 | 13.5 ± 1.2 | 4.1 ± 0.4 | 1.8 ± 0.5$^1$ |

Values are the means (±SD) of the percentages obtained in 4 independent experiments. In each experiment at least 20 randomly chosen root tips per genotype and developmental stage were analysed.

$^1$ These values are statistically different (P<0.01)
Table VI  Ploidy level distribution in wt and gte4 mutant plants.

|       | 3-DAYS ROOTS |     | 4-DAYS ROOTS |     |
|-------|--------------|-----|--------------|-----|
|       | wt           | gte4| wt           | gte4|
| 2c    | 44.3 ± 2.2   | 45.5 ± 2.9  | 2c           | 46.1 ± 3.3 | 34.8 ± 2.1 *|
| 4c    | 43.8 ± 2.8   | 43.1 ± 3.3  | 4c           | 42.5 ± 4.2 | 43.4 ± 1.4|
| 8c    | 11.9 ± 1.7   | 11.4 ± 1.9  | 8c           | 11.4 ± 2.1 | 21.8 ± 3.8 *|
| 16c   | n.d.         | n.d.        | 16c          | n.d.       | n.d.        |
| 32c   | n.d.         | n.d.        | 32c          | n.d.       | n.d.        |

|       | 5-DAYS ROOTS |     | 10-DAYS ROOTS |     |
|-------|--------------|-----|---------------|-----|
|       | wt           | gte4| wt            | gte4|
| 2c    | 41.9 ± 2.7   | 33.3 ± 2.6 *| 2c           | 36.8 ± 2.7 | 22.7 ± 2.1 *|
| 4c    | 43.5 ± 1.9   | 45.2 ± 2.1  | 4c           | 27.4 ± 1.9 | 31.3 ± 1.6 *|
| 8c    | 14.6 ± 1.9   | 21.5 ± 1.8 *| 8c           | 30.7 ± 1.7 | 36.6 ± 2.1 *|
| 16c   | n.d.         | n.d.        | 16c          | 5.1 ± 1.4  | 9.4 ± 0.6 * |
| 32c   | n.d.         | n.d.        | 32c          | n.d.       | n.d.        |

|       | 30-DAYS ROOTS |     | FIRST IMMATURE LEAVES |     |
|-------|---------------|-----|-----------------------|-----|
|       | wt            | gte4| wt                     | gte4|
| 2c    | 32.3 ± 2.3    | 24.3 ± 2.6 *| 2c           | 74.2 ± 3.8 | 75.2 ± 3.4|
| 4c    | 24.9 ± 1.9    | 24.7 ± 2.3  | 4c           | 25.1 ± 1.9 | 23.9 ± 1.6|
| 8c    | 34.2 ± 2.1    | 40.4 ± 2.1 *| 8c           | 0.7 ± 0.4  | 0.9 ± 0.6 |
| 16c   | 8.4 ± 0.9     | 9.9 ± 0.9   | 16c          | n.d.       | n.d.        |
| 32c   | 0.1 ± 0.06    | 0.7 ± 0.1 * | 32c          | n.d.       | n.d.        |

|       | DIFFERENTIATED LEAVES |     | COTYLEDONS |     |
|-------|-----------------------|-----|------------|-----|
|       | wt                     | gte4| wt         | gte4|
| 2c    | 49.9 ± 1.8            | 44.2 ± 2.1 *| 2c        | 42.6 ± 2.1 | 30.2 ± 2.4 *|
| 4c    | 42.2 ± 1.6            | 36.5 ± 2.1 *| 4c        | 39.9 ± 2.7 | 36.3 ± 2.6|
| 8c    | 6.3 ± 1.7             | 13.8 ± 1.3 *| 8c        | 13.5 ± 1.7 | 26.3 ± 2.2 *|
| 16c   | 1.4 ± 0.8             | 5.2 ± 1.6 * | 16c       | 2.8 ± 1.0  | 5.2 ± 1.1 * |
| 32c   | 0.2 ± 0.1             | 0.3 ± 0.1  | 32c        | 1.2 ± 0.6  | 2.0 ± 0.7 |

Pooled plantlets or single mature plants subjected to flow cytometric analysis according to organ type at successive times from the beginning of seed imbibition. The reported values are the means (± SD) of the percentages obtained in 4 independent experiments. In each experiment, at least 5 pools of about 100 plantlet leaf primordia, 5 pools of 10 cotyledons and at least 5 pools of about 50
roots were analysed (3, 4, 5 and 10 days old plantlets); in each experiment about twenty 30-days-old plants were instead individually analysed to determine ploidy in mature leaves and roots.

*Statistically different (P<0.01); n.d. non determined
Legends

Figure 1. Phylogenetic analysis of the Arabidopsis BET Bromodomain family. Rooted tree obtained with a clustal X alignment and subsequent analysis with Phylyp protdist neighbour joining, the alignment was made considering only the bromodomain (of human BRD4 only the second bromodomain was considered). The numbers represent the branching reproducibility over hundred bootstrap.

Figure 2. Analysis of the gte4 mutant. A, Schematic representation of T-DNA insertion in GTE4. B, Differences in inflorescence height in wild-type and the gte4 mutant. C, Rosettes of the gte4 mutant are smaller than those of wt plants. D, Comparison of leaf size and shape between the gte4 mutant and wt. Gte4 leaves are visibly more jagged than those of wt. E, Transections of wt and gte4 stem internodes showing the reduced stem thickness in gte4 plants (55 days after germination, unstained sections under light microscopy; bars = 50 µm). F, wt and gte4 flowers at anthesis. The stamen number (arrows) in gte4 flower is reduced in comparison with wt (Bars = 0.5 mm).

Figure 3. Effects of gte4 mutation on root development in young seedlings. A and B, young seedlings; C and D, primary root; E-L, lateral root apices. A, wt and B, gte4 unstained sections under light microscopy of 4 days old seedlings. The gte4 primary root is shorter than the one of wt due to a reduction in length in the hairy region (h.r.) and elongation region (e.r.) (Bars = 100µm). C, wt and D, gte4 apices of the primary root of seedlings at day 4 after germination showing the different length of the apical region (brackets). (Bars = 10 µm). Magnifications of the quiescent center cells (QC) and surrounding initial cells are shown in the insets. The arrows in the inset of D show anomalous anticlinal divisions in the procambial cells. (Bars = 10 µm). E and F, Unstained sections under light microscopy showing details of the primary root of wt (E) and gte4 (F) 9-days-old-seedlings revealing a lower density, and a reduced development, of lateral roots (arrows) in the mutant. (Bars = 200 µm). G and H, wt (G) and gte4 (H) lateral root apices at early emergence from the primary root in 12- days-old seedlings showing apical initials and QC cells equally defined in both genotypes. (Bars=10 µm). I and J, details of wt (I) and gte4 (J) lateral root apices during the expansion phase of the emerged organ in 14 days-old-seedlings showing that expansion in the QC cells and columella initials is higher in gte4 roots than in the wild-type (squares) (Bars=10 µm). K and L, apices of protruded lateral roots in wild-type (K) and gte4 (L) plants. Rectangles show QC cells, columella and procambial initials in the gte4 mutant in comparison with wild-type. A magnification of hardly visible QC cells and of irregularly sized surrounding cells is shown in the inset (Bars=10 µm). (C-D and G-L, images by Nomarski microscopy). QC cells are marked with asterisks.
**Figure 4.** Effects of the *gte4* mutation on embryo development. Panels on the left are from wild-type and those on the right are from the *gte4* mutant. A-D, in the *gte4* mutant there is no visible effect on embryo development at the octant stage (A and B) and heart stage (C and D), whereas the suspensor cells are enlarged (Bars= 10µm). E and F, wild-type (E) and *gte4* (F) embryos at the cotyledonary stage showing no significant alteration in the cotyledons (Bars= 50µm). G and H, details of the root pole of the embryo at the cotyledonary stage showing irregularly shaped QC cells (arrows), and irregularly divided columella initials (brackets) in the mutant (Bars= 10µm). I and J, details of wild-type and *gte4* apices of mature embryos showing a different shape in the mutant (Bars = 10 µm). A-H, longitudinal toluidine blue stained sections of developing seeds observed under light microscopy. I and J, images by Nomarski microscopy after 5 h of imbibition on filter paper.

**Figure 5.** Expression of QC markers in the root apex of wild-type (A, C, and E) and *gte4* seedlings (B, D, and F). A and B, expression of AGL42-GFP in the apex of the primary root. A, wt root showing a strong GFP signal in the QC cells (Higher magnification in the inset). B, absence of GFP signal in the root apex of a *gte4* mutant containing the same construct. (Bars=20µm). C to F, expression of the QC46 (C and D) and QC25 (E and F) GUS reporter in the QC cells of wt and *gte4* mutant roots (Bars=10 µm).

**Figure 6.** Quantitative reverse transcription PCR analysis of genes that are involved in the E2F pathway. The graphs show the relative expression of each gene in *gte4* mutant compared to the WT. Error bars indicate standard errors. RNA was extracted from 4-days-old Arabidopsis plantlets.

**Supplemental Figures:**

**Figure S1.** *GTE4* expression analysis by reverse transcription PCR. RNA extracted from R (roots), L (leaves), St (stems), F (flowers) and S (siliques) was used for this analysis. The transcript is present in all the tissues analyzed.

**Figure S2.** Reverse transcription PCR expression analysis of *GTE4* in wild-type and *gte4* mutant plants. The *GTE4* transcript is not detected in the mutant. *ACT* is the actin control.
Figure S3. DAPI and BrdU staining analysis on wt and gte4 mutant roots. A and B, wild-type roots. C and D, mutant roots 72 hours after germination. E and F, non-germinated mutant roots. A, C and E, DAPI staining B, D and F BrdU staining.
