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Expression of *Arabidopsis APETALA1* in tomato reduces its vegetative cycle without affecting plant production

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Abstract

Important agronomic traits such as fruit quality, harvesting efficiency or production largely depend on flowering time. We have analysed the effect of the overexpression of the *Arabidopsis APETALA1* MADS-box gene on vegetative and reproductive growth of tomato. Constitutive expression of *APETALA1* in tomato plants has major effects on the length of their growth cycle as well as on their growth habit. Transgenic tomato plants initiated flowering after the production of 6 vegetative nodes as compared to 11 nodes for the wild type plants. Most of tomato *35S:AP1* plants also showed determinate growth habit, similar to the phenotype of *self pruning* tomato mutants, as well as an initial reduction of their axillary growth. Moreover, development and fertility of flowers were not affected in plants expressing *AP1*. Consequently, fruit formation in transgenic plants grown under greenhouse conditions occurred normally, which permitted a similar fruit yield compared to control plants. Since traits conferred by *AP1* expression are dominant, its expression in tomato breeding lines could provide advantages for the development of new hybrid varieties with shorter generation time, determinate growth, and reduced pruning requirements.

Introduction

The length of growth cycle as well as plant architecture are important agronomic traits in many plant species. The length of growth cycle is one of the most limiting factors for the production of specific varieties in Northern and Southern latitudes and also affects the number of production cycles in more temperate or tropical latitudes. Additionally, plant architecture greatly influences cultural practices and the possibility of mechanized harvests in many crop species (see for example Stevens and Rick 1986; Wang et al. 1999). Extensive breeding in the last century has reduced the growth cycle of the cultivated tomato plants and generated varieties whose reproductive biology is independent of environmental conditions (Atherton and Harris 1986). Regarding plant architecture, tomato has a sympodial growth habit since vegetative

and reproductive phases alternate regularly in the adult plant (Atherton and Harris 1986¸ Sawhney and Greyson 1972). Mutations at the *SP* gene have commonly been used to generate tomato cultivars with determinate growth, most of them for the processing industry (see Atherton and Harris 1986; MacArthur 1932). In these cultivars, sympodial segments progressively develop fewer vegetative nodes until the shoot is terminated by two consecutive inflorescences. Although *sp* mutation has been very useful and is nowadays used for breeding purposes, its recessive nature makes the rapid development of new hybrid cultivars more difficult since the *sp* allele must be introgressed in both parental lines. It is noteworthy that commercial hybrids are particularly valuable both for fresh and processing tomato varieties given the increasing agronomic requirements that they must satisfy (Grandillo et al. 1999).

Genetic and environmental factors controlling growth habit and plant architecture have begun to be elucidated thanks to extensive studies carried out in the model species *Arabidopsis thaliana* (Bradley et al. 1997; Mouradov et al. 2002; Simpson and Dean 2002). In this monopodial species, flower meristem identity genes such as *LEAFY* (*LFY*) and *APETALA1* (*AP1*) have been shown to be not only required but sufficient to promote flowering (Mandel and Yanofsky 1995; Weigel and Nilsson 1995). Furthermore, ectopic expression of *LFY* or *AP1* in the shoot apical meristem of *Arabidopsis* causes the production of terminal flowers in a similar way as the phenotype caused by mutations in the *TERMINAL FLOWER1* gene (Bradley et al. 1997) which is orthologous to the tomato *SP* gene (Pnueli et al. 1998). Constitutive expression of either *LFY* or *AP1* in other herbaceous and woody species has also been shown to accelerate the initiation of flowering (He et al. 2000; Peña et al. 2001; Rottman et al. 2000; Weigel and Nilsson 1995) strongly suppressing the juvenile phase in some woody species (Peña et al. 2001; Weigel and Nilsson 1995). Comparisons between different plant systems (monopodial and sympodial, herbaceous and trees) seems to demonstrate that all share a set of regulatory genes needed for flower initiation although the genes may act through different pathways and specific regulatory gene interactions (see for example Amaya et al. 1999; Molinero-Rosales et al. 1999; Peña et al. 2001; Pnueli et al. 1998). This feature has opened the possibility to improve some traits of agronomical importance by constitutive expression of heterologous genes.

In this work, we analysed the possibility of generating tomato cultivars with shortened life cycle and determinate growth by overexpressing the *Arabidopsis AP1* gene. The results show that in tomato, a sympodial species of great horticultural interest, overexpression of *AP1* causes a significant reduction of the growth cycle due to a shortening of the length of the vegetative phase preceding the production of the first inflorescence and a reduction in the number of sympodial segments. Transgenic tomato plants also showed a 'determinate' growth habit and a similar fruit production when compared to non-transformed controls. Taking into account that new traits conferred by *AP1* expression, i.e., earliness and determinate growth are dominant, they could be useful in the generation of improved hybrid cultivars of tomato, both for fresh and processing markets.

Materials and methods

Plant material and growth conditions

Tomato (*Lycopersicon esculentum* Mill.) cv. p73, a breeding line kindly provided by Dr. M.J. Diez (UPV, Valencia, Spain), was used in this work. Seeds were surface sterilised and germinated in darkness on solid germination medium (GM) consisting of MS salt solution (Murashige and Skoog 1962) supplemented with 1% (w/v) sucrose. After 3 to 4 days, when the radicle emerged and curved into the medium, tubes containing germinated seeds were transferred to a tissue culture chamber at 24 °C ± 2 °C. Plants were grown in pots under natural photoperiod and greenhouse conditions. Both transgenic and control plants were self-pollinated and seeds were harvested from mature fruits for progeny analyses.

AP1 plasmid construction and Agrobacterium-mediated transformation

The cDNA sequence (1.2 kb fragment) of the *Arabidopsis APETALA1* (*AP1*) gene (Mandel et al. 1992) was cloned into the *Sma*I restriction site of vector pROKII (Baulcombe et al. 1986) under the control of the Cauliflower Mosaic Virus 35S promoter (CaMV 35S). The construct was verified by DNA sequencing and its functionality tested on transgenic *Arabidopsis* plants. This vector was electroporated into *Agrobacterium tumefaciens* LBA 4404 strain for further use in genetic transformation experiments. Bacteria were

grown overnight in LB medium (100mL flask) with rifampicin 40 mg L^{-1} and kanamycin 50 mg L^{-1}. Cotyledons from 11-12 day-old seedlings were transversally cut in two segments and placed in Petri dishes containing preculture medium, that is a shoot induction medium without zeatin. After 2 days in the dark, *Agrobacterium*-mediated transformation was performed following the protocol described by Ellul et al. (2003).

Molecular analysis of transgenic plants

Standard PCR reactions were performed to detect the presence of the *nptII* selectable gene and the *Arabidopsis AP1* gene in putative transgenic tomato plants originated from independent cotyledon explants. PCR reactions with *nptII*-forward (5'-AAGATGGATTGCACGCAGGTTC) and *nptII*-reverse (5' GAAGAACTCGTCAAGAAGGCGA) primers amplified a fragment of 781 bp from the selectable gene (Beck et al. 1982). *AP1* specific primers used, AP1-F (5'-ATGGGAAGGGGTAGGGTTCAATT-3') and AP1-R (5'-TGCGGCGAAGCAGCCAAGGTT-3'), generated a 768 bp fragment covering the complete cDNA sequence of *AP1*. In both cases, reactions consisted of 5 minutes at 94 °C and 30 cycles including 30 seconds at 94 °C, 1 min at 55 °C and 1 min at 72 °C. To determine the copy number of *AP1* gene in each tomato transgenic line, a Southern blot hybridisation experiment was performed from 10 micrograms of genomic DNA digested with restriction enzymes *Eco*RI, *Bam*HI and *Hin*dIII and hybridised with the full-length cDNA of *AP1* following described protocols (Ausubel et al. 1995). Expression of the *AP1* transgene was analysed by RNA gel-blot hybridization using total RNA isolated from leaves (Nagy et al. 1988).

Analysis of ploidy level

Ploidy level of individual plants was measured by flow cytometry according to Smulders et al. (1995) with some modifications. Leaves from primary transformants cultivated on rooting medium (antibiotic free), were used for nuclei isolation. Pieces of leaf tissue (1 cm^2) were chopped individually on a 50 mm glass plate with 200 μL of nuclei isolation buffer per sample. After chopping, the resuspended sample was passed through a 50 μm nylon filter, and 800 μL of DAPI solution (1 mg L^{-1}) was added for fluorescent DNA staining. The DNA content of the isolated nuclei was measured using a Partec PAS-II flow cytometer equipped with a mercury lamp. About 5.000 to 10.000 nuclei were measured per sample.

Analyses of agronomic traits and greenhouse performance of transgenic tomatoes

Agronomic trait evaluation was performed under controlled greenhouse conditions over 4 months. The trial included the cultivation of 3 replicates of 8 independent primary transformants expressing the *AP1* gene and 12 non-transformed tissue culture regenerated control plants.

In order to evaluate the effects of the constitutive expression of *Arabidopsis AP1* gene on the vegetative growth features of tomato, plants were scored at 5, 8, 13 and 18 weeks after acclimation to greenhouse conditions. In addition, growth of axillary buds was measured 5 and 8 weeks after transferring plants to the greenhouse. We also assessed the effects of *AP1* overexpression on the shoot apical meristem and floral transition of tomato. With this purpose, we determined the number of plants showing a conversion of the shoot apical vegetative meristem into an inflorescence or floral meristem. The flowering time was estimated as the number of leaves (nodes) developed before the first inflorescence. Similarly, with the aim of comparing fruit yield of *AP1* transgenic and untransformed p73 tomato plants, red fruits were harvested 14, 15, 16 and 17 weeks after the beginning of the greenhouse trial. Three different variables were measured to analyse yield: total fruit weight per plant (g), number of fruits per plant and fruit mean weight (g).

Results

Expression of AP1 in transgenic tomato plants

Transgenic tomato plants (cv. p73) constitutively expressing the *Arabidopsis AP1* gene were generated by *Agrobacterium*-mediated transformation with one *AP1* construct containing the coding sequence of the *AP1* gene (Mandel et al. 1992) fused to the CaMV 35S promoter and the nopaline synthase 3'-end sequences (Baulcombe et al. 1986). Regenerating tomato plants were evaluated for their ploidy level by flow cytometry. Out of 35 kanamycin resistant transformants only 8 transgenic plants were diploid while the remaining 27 were tetraploid. All diploid plants

Table 1. Expression level of *AP1* and segregation analysis of the *nptII* marker gene in progenies from self-pollinated tomato transgenic plants. Data were obtained after 4 weeks of culture on rooting medium supplemented with 50mg.l^{-1} kanamycin. All p73 untransformed (control) plants were sensitive under the same conditions (not shown).

Plant	Expression level of AP1[#]	Observed segregation			χ^2 Value *	P
		N	kan^R	kan^S		
241	+++	106	84	22	1.019	0,25 < P < 0,50
242	+	137	0	137	all sensitive	–
243	+++	239	184	55	0.503	0,50 < P < 0,75
244	+	161	0	161	all sensitive	–
441	+++	45	33	12	0.067	0,90 < P < 0,95
413	++	90	62	28	1.790	0,10 < P < 0,25
414	+	70	48	22	1.540	0,10 < P < 0,25
465	+	128	95	33	0.042	0,75 < P < 0,90

[#]Relative levels of *AP1* transcript; N: number of seedling plants tested; kan^R= kanamycin-resistant; kan^S= kanamycin sensitive; * = Chi-square value for an expected 3:1 segregation; *P*= probability of a greater $\chi^2$for one degree of freedom.

were rooted on the selective medium and were found PCR positive for both *nptII* and *AP1* genes. In addition, the copy number of *AP1* gene in each diploid transgenic line was determined by Southern blot hybridisation analysis, and this indicated that all primary transformants carried a single copy of the transgene (data not shown). Diploid transformants appeared to be phenotypically normal either during the organogenic callus phase or the propagation phase. Nevertheless, four plants produced flower buds under *in vitro* culture conditions. This feature had never been observed in non transgenic p73 plants regenerated *in vitro*, and did not seem to be related to the expression level of *AP1* transgene in plants regenerated subsecuently (see below).

Expression of *AP1* gene in transgenic plants was confirmed by RNA blot hybridisation performed on RNA samples isolated from leaves. All diploid transformants showed significant levels of *AP1* transcripts indicating that *AP1* was indeed expressed in the leaves of transgenic tomato plants (Table 1). When compared to the absolute absence of *AP1* expression in the non-transformed plants, significant expression levels were observed among the transgenic plants, ranging from high (lines 241, 243 and 411) to low *AP1* transcript levels (lines 242, 244, 414 and 465). Diploid transgenic plants positive for AP1 by PCR, Southern and Northern blot analyses were considered for further analyses. Stability and mendelian inheritance of the kanamycin resistance gene were analysed from T2 progenies obtained by selfing the 8 independent primary transformants (Table 1). The expected ratio for a single T-DNA insertion locus (3 kanamycin resistant: 1 kanamycin susceptible) was observed for 6 genotypes although for transformants 413 and 414 the progeny test could better fit a 2:1 segregation rate. This could suggest a reduced viability of homorygous transgenic plants carrying T-DNA insertion. The progenies of two transgenic plants expressing the *AP1* gene (242 and 244) were completely sensitive to kanamycin even though presence of the *nptII* gene has been confirmed by PCR (Table 1). Southern blot analysis confirmed the presence of *AP1* in the genome of these T2 plants indicating that they had lost the *nptII* gene after selection in tissue culture (Iglesias et al. 1997; Kooter et al. 1999).

Expression of AP1 promotes early flowering

The primary shoot of cv. p73, as other tomato cultivars, develops the first inflorescence after the production of 10-12 leaves. Growth then continues from the uppermost axillary bud below the inflorescence, which generates 3 more leaves before terminating with a new inflorescence. New emerging vegetative shoots develop regularly following this simpodial pattern. In order to study the effect of *AP1* overexpression on the morphological characteristics of p73 tomato plants, transformants were grown under greenhouse conditions together with the appropriate controls, i.e., non-transformed plants independently regenerated by *in vitro* culture. A primary effect of *AP1* expression in tomato plants was a shortening of flowering time as measured by the number of leaves (nodes) developed before the formation of the first inflorescence (Figure 1). Transgenic plants flowered after the production of an average of six leaves (6.3 $\pm$ 0.6) while control plants produced more than

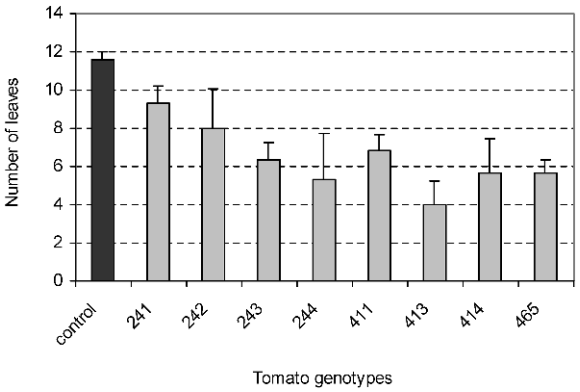

Figure 1. Effect of the constitutive expression of the *Arabidopsis AP1* gene on the flowering time of cv.p73 tomato plants. The number of leaves before the development of the first inflorescence was assessed for each tomato genotype after 18 weeks of culture in greenhouse conditions. Bars represent the standard error of the mean.

eleven leaves before flowering (11.6 ± 1.4). Apart from this early flowering phenotype, transgenic tomato plants did not display alterations in the number of leaves between consecutive inflorescences in the other sympodial segments analysed.

Tomato plants expressing *AP1* also showed an early conversion of the shoot apical meristem into an inflorescence or a single terminal flower yielding a determinate phenotype (Figure 2 A and B). After 18 weeks of culture under greenhouse conditions, the percentage of transgenic plants exhibiting a floral conversion of the apical meristem was more than two-fold higher (68.9%) than in control plants (30%). The latter result is in agreement with the growth habit of the cultivar (p73) used as control and probably indicates an incomplete penetrance of this trait in this genotype. Axillary meristems of transgenic plants never transformed into floral meristems and developed normally generating vegetative shoots although their growth before flower transition was slower than in the control plants (Figure 2 C and D). Furthermore, as a consequence of the determinate growth habit, a slight decrease in the average number of inflorescences was observed in transgenic plants (9.00 ± 0.49) with respect to controls (10.07 ± 0.36) when this parameter was measured at 18 weeks. Transgenic plants did not show significant differences with respect to non-transformed controls in the number of flowers per inflorescence nor in the identity of floral organs.

Expression of AP1 in transgenic tomato plants does not affect fruit yield

All transgenic plants developed fertile flowers and regular fruits. Both the size and shape of the transgenic fruits were similar to those of control fruits. Furthermore, all transgenic fruits showed a regular ripening process. The only morphological difference was the higher number and smaller size of the locules observed in transgenic fruits (Figure 3). Seeds from transgenic fruits were completely fertile and germinated in the same proportion than seeds from control fruits. In order to ascertain whether the early flowering and determinance traits would affect the final yield, a comparative trial was performed among transgenic and control regenerated plants, under greenhouse conditions. Three different variables were measured, fruit weight, number of fruits per plant and total weight production (Table 2). The results indicated that the fruit mean weight was not significantly different (92.5 ± 4.6 g and 97.6 ± 5.4 g, in transgenic and control plants, respectively) although overall yield measured as total fruit weight per plant was slightly lower in transgenic tomato plants since they produced on average two fruits per plant less than control plants. Expression levels of *AP1* gene did not seem to be correlated with the yield variables analysed despite the fact that transgenic line 244, that showed a high *AP1* expression level and a very early phenotype (see Figure 1), was also found to produce a significantly greater yield than controls (Table 2).

Discussion

We have analysed the effects of constitutive expression of *AP1* on vegetative and reproductive growth of tomato. With this aim, we have selected diploid transgenic plants carrying a single copy of the transgene. Tomato plants expressing the *AP1* transcript show a significant reduction on their time of flower initiation from the 11-12th node to the 6th node. This effect demonstrates that *AP1*, which functions as a developmental switch in *Arabidopsis*, can also play a similar role in heterologous systems like tomato. *AP1* does not alter inflorescence or flower development. Similar results were described for *AP1* in citrus (Peña et al. 2001), which indicates that this could be a general effect of *AP1* heterologous expression. Constitutive expression of *AP1* also promotes determinate growth habit in transgenic tomato plants in a similar

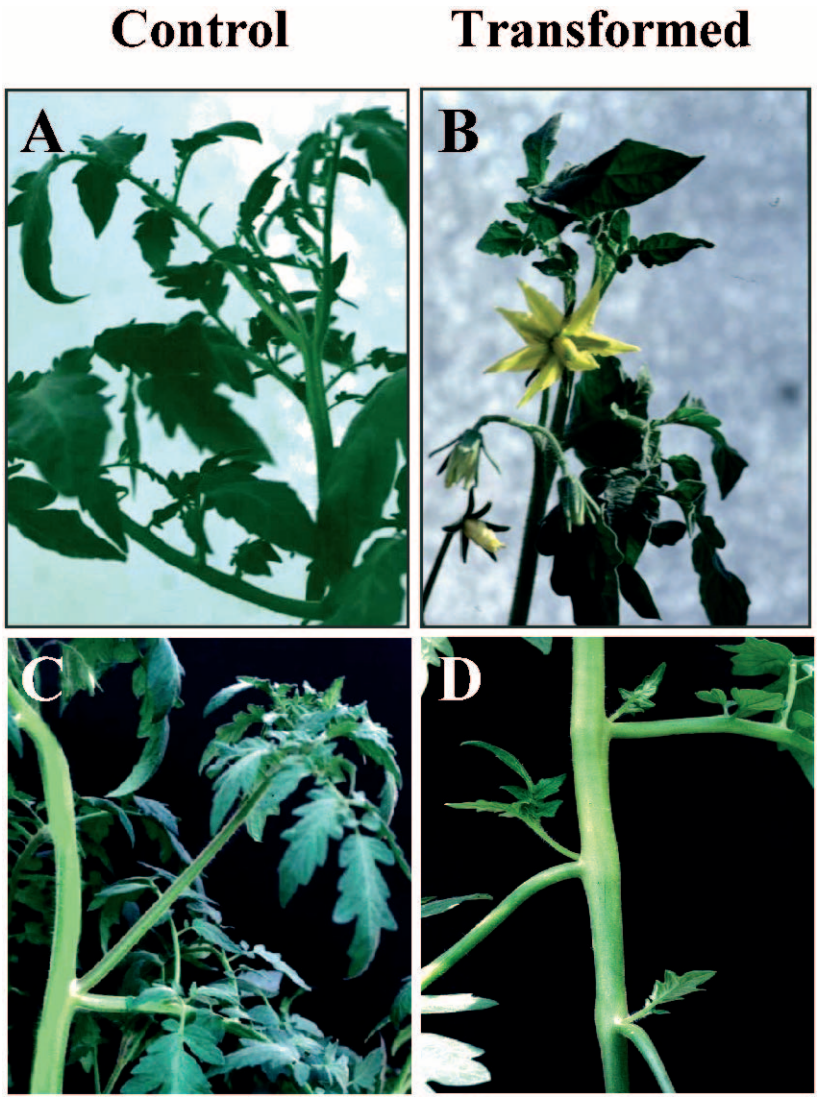

Figure 2. Effect of the constitutive expression of the *Arabidopsis AP1* gene on the development of tomato plants. Compared to the indeterminate growth displayed by tomato control plants (A), transformant lines showed an early conversion of the apical meristem into a floral meristem, and consequently a determinate growth habit (line 441 in B). Before flowering, growth of the lateral branches in non-transformed plants (C) was faster and greater than in transgenic plants (line 243 in D).

way to the effects of the *sp* mutation. However, transgenic plants differ from *sp* mutants in two major features. First, mutant plants at the *SP* locus do not show an acceleration of flowering time and initiate the first inflorescence at the same node as wild type plants. Second, the development of the sympodial segments after the first inflorescence does not seem to be affected in *35S:AP1* transgenic tomatoes whereas, in *sp* mutants, they show a progressive reduction in their node number till the last sympodial meristem is converted into a floral meristem (Atherton and Harris 1986; MacArthur 1932; Yeager 1927).

The *sp* allele has been introduced into a great number of tomato lines for breeding purposes since it permits synchronous ripening of fruits and mechanical harvest (see Atherton and Harris 1986). During the last two decades tomato breeding programs have focused on the development of F_1 hybrid varieties since they are more profitable as a consequence of heterosis and intellectual property rights. Nevertheless, the introgression of a new trait controlled by a

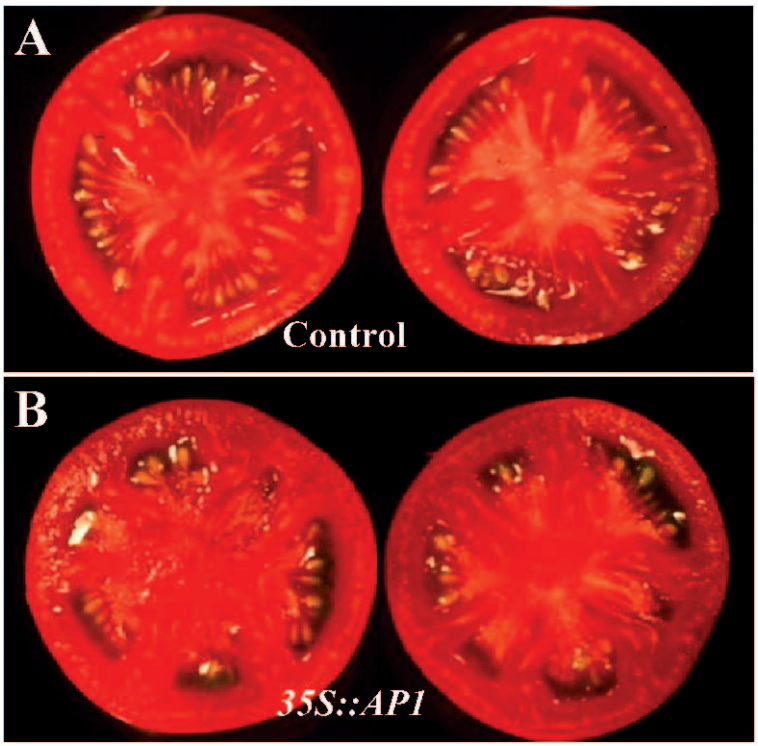

Figure 3. Both tomato fruits produced by control (A) and *AP1* transgenic plants (line 244 in B) were fertile and showed similar size, weight and shape. The only morphological difference was the high number and smaller size of locules developed in transgenic fruits. See Results section for further details.

Table 2. Fruit yield in *AP1* transgenic and untransformed p73 tomato plants. Red mature fruits were harvested at 14, 15, 16 and 17 weeks after the beginning of the greenhouse trial.

Plant genotype[#]	AP1 expression level	Number of fruits per plant ± SE	Fruit mean weight (g) ± SE	Total fruit weight per plant (g) ± SE
241	+++	16.3 ± 1.5	71.1 ± 19.7	1439 ± 107
242	+	14.3 ± 2.2	88.0 ± 8.5	1737 ± 197
243	+++	14.0 ± 1.5	102.7 ± 1.6	1442 ± 100
244	+	23.0 ± 1.0	100.0 ± 9.0	2492 ± 121
411	+++	15.0 ± 2.1	108.7 ± 13.7	1834 ± 202
413	++	16.0 ± 3.8	80.3 ± 23.2	1424 ± 205
414	+	17.7 ± 1.9	102.6 ± 9.3	1967 ± 161
465	+	16.1 ± 0.6	87.3 ± 23.2	1620 ± 151
Mean of 35S: AP1 plants		16.3 ± 1.1 [n.s]	92.5 ± 4.6 [n.s]	1744 ± 128 [n.s]
Mean of control plants		18.4 ± 1.9 [n.s]	97.6 ± 5.4 [n.s]	2059 ± 169 [n.s]

[#]Three replicates of 8 independent primary transformants expressing the *AP1* gene and 12 non-transformed control plants were used; SE = standard error of the mean; n.s. No statistically significant differences.

recessive gene such as *sp* requires that crosses and selection strategies must be performed from both parental lines which complicates the breeding process. Our results show that determinance and earliness can be generated as dominant traits by overexpression of *AP1* in transgenic tomato plants. Thus, the use of *AP1* plants as donors could be a reliable choice to introduce both traits into new commercial hybrids.

Once initiated, the main shoot apex regulates further development of axillary buds into lateral shoots. This phenomenon of apical dominance is known to have a weak inhibitory effect on the axillary shoots

of tomato in such a way that these are initiated early and develop into side-shoots without a resting phase (Malayer and Guard 1964; Tucker 1977). Nevertheless, transgenic tomato plants overexpressing *AP1* show a reduced development of axillary shoots during the first stage of vegetative growth, when flowering has not yet been induced. Later, the initiation of flowering seems to promote a progressive loss of the apical dominance which finally disappears in parallel with the change of apical meristem identity, from vegetative to floral. At this moment, branching and vigorous growth of side shoots were observed in transgenic plants, together with the determinate growth habit phenotype. Interestingly, *lateral suppressor* (*ls*) mutant plants of tomato display a similar phenotype. They lack side-shoots during vegetative growth but at the time of transition to reproductive development, axillary meristems are initiated in the axils of two leaves preceding the inflorescence (Malayer and Guard 1964; Schumacker et al. 1999).

Constitutive overexpression of *AP1* in tomato significantly reduces the time to flowering without greatly affecting the total yield. The flowers produced by transgenic plants are fertile and produce fruits of equivalent average weight compared to control plants. From an economic point of view, the small reduction in the number of fruits per plant could be compensated by a shorter production period. Bearing in mind these features of *35S:AP1* tomato plants, as well as the mendelian inheritance of the *AP1* gene, the use of this plant system could improve the development of new short-cycle varieties in temperate regions where several crops per year could be harvested. Indeed, tomato production is currently concentrated in such regions since growing is favoured by environmental conditions. On the other hand, the availability of short-cycle hybrids would also be particularly interesting in those areas of extreme climate where tomato crop production is limited to the short warm season.

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