Complementation of *notabilis*, an abscisic acid-deficient mutant of tomato: importance of sequence context and utility of partial complementation

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ABSTRACT

The abscisic acid (ABA)-deficient tomato mutant *notabilis* (*not*) is believed to be a null mutation in the gene *LeNCED1*, encoding a 9-cis-epoxycarotenoid dioxygenase involved in ABA biosynthesis. We have sequenced and analysed a 19 kb genomic clone containing *LeNCED1* and 5.4 kb of its promoter. This clone was transferred to *not* homozygotes and several non-wilty transformed plants were obtained. The basal ABA content, water relations, shoot and root growth, adventitious rooting, ethylene evolution and ability to accumulate ABA under water stress are described for two of these lines, *not* comp.1 and *not* comp.1*. Partial complementation was observed for most parameters measured for *not* comp.1. Full complementation was observed in *not* comp.1 for all parameters measured in whole plants under well-watered and water-stressed conditions. These data provide further evidence that *LeNCED1* is the wild-type allele of the *not* mutant gene. However, *not* comp.1 was unable to accumulate the wild-type levels of ABA in rapidly dehydrated leaves, indicating that it too was only partially complemented. Since *LeNCED1* is an environmentally regulated gene encoding a rate-limiting enzyme, precise levels and patterns of gene expression may be needed to fully recreate wild-type phenotype. The utility of partially complemented lines to study the role of ABA in plant responses to stress conditions, and in promoter analysis, is discussed.

Key-words: 9-cis-epoxycarotenoid dioxygenase; growth; matrix attachment region; promoter; tomato; water stress.

INTRODUCTION

The tomato mutants *notabilis* (*not*), *flacca*, and *sitiens* are deficient in abscisic acid (ABA) and are phenotypically wilty, stunted and epinastic (Tal 1966). The poor growth and strong leaf epinasty in these mutants occurs even under non-wilting conditions, and has been attributed, at least partially, to an excess of ethylene (Sharp et al. 2000). It has been proposed that restriction of ethylene production may be a widespread function of ABA, and that, as a result, ABA may often function as a promoter of growth (Sharp 2002; Sharp & LeNoble 2002). However, the role of increased ABA levels in shoot growth during abiotic stresses, such as soil compaction and water stress, remains controversial with evidence for both positive (Mulholland et al. 1996, 1999; Sharp 2002) or negative (Creeelman et al. 1990; Saab et al. 1990; Zhang & Davies 1990) effects of ABA on growth. The use of ABA-deficient mutants to study the role of ABA in shoot growth under stress has proven difficult because of their adverse water relations and aberrant growth under non-stressed conditions. An ideal genotype to study the role of ABA in stressed plants would be one in which ABA content (and therefore water relations, ethylene and growth phenotype) is normal under non-stressed conditions, but in which ABA content fails to increase in response to stress.

Four genes encoding enzymes in the ABA biosynthetic pathway have now been cloned: zeaxanthin epoxidase (ZEP) (Marin et al. 1996), 9-cis-epoxycarotenoid dioxygenase (NCED) (Schwartz et al. 1997), xanthoxin oxidase (Rook et al. 2001; Cheng et al. 2002; Gonzalez-Guzman et al. 2002), and ABA aldehyde oxidase (AAO) (Seo et al. 2000). These genes, together with the availability of ABA-deficient mutants, particularly in tomato and *Arabidopsis thaliana*, now offer many possibilities for the creation of new genotypes with altered patterns and rates of ABA biosynthesis. Over-expression of ZEP in tobacco has been shown to increase seed ABA content and prolong seed dormancy (Frey et al. 1999). When the tomato NCED gene *LeNCED1* was expressed in tomato plants using a constitutive promoter, increased leaf and seed ABA content were observed along with reduced leaf transpiration and increased seed dormancy (Thompson et al. 2000b). Similar results from over-expression of NCED genes have been obtained in *Arabidopsis* (Iuchi et al. 2001) and tobacco.
(Qin & Zeevaart 2002). These studies demonstrate that both ZEP and NCED are rate-limiting enzymes in ABA biosynthesis, although, in the case of ZEP, this may only be true in non-green tissues in which the product of this enzyme, violaxanthin, is not already in abundance (Parry & Horgan 1992). NCED may be an important regulatory enzyme in a wide range of tissues as it represents the first dedicated step in ABA biosynthesis. NCED mRNA is known to increase during dehydration stress in both roots and leaves of tomato (Thompson et al. 2000a), in leaves of bean (Qin & Zeevaart 1999) and maize (Tan et al. 1997) and also during ripening of Avocado fruit (Chernys & Zeevaart 2000). Nevertheless, further studies are required to establish how gene expression is regulated during other aspects of plant development and stress.

It was previously shown that the not mutation is closely linked to gene LeNCED1, and that the allele of LeNCED1 present in the not mutant is likely to be inactive due to a frame-shift mutation (Burbidge et al. 1999). This, together with evidence from over-expression of LeNCED1 in tomato described above, strongly suggests that the not gene is equivalent to LeNCED1. Further evidence of the identity of the not gene can be obtained through genomic complementation of the mutation. Here we describe the isolation and characterization of a genomic clone containing LeNCED1 and its promoter, and its use to achieve varying degrees of complementation of not. The value of partially complemented ABA-deficient mutants in studies of the role of ABA in stress responses and in promoter analysis is discussed.

MATERIALS AND METHODS

Sequencing

The 19 kb cosmids clone pNCED1.1 was mapped by restriction enzyme digestion, and four plasmid subclones were created encompassing the entire clone. Each subclone was sequenced by primer walking. Sequencing reactions (Applied Biosystems (ABI), Warrington, UK; BigDye v2 or v3) were analysed with an ABI PRISM 377 automated sequencer. Sequence ambiguities were resolved by sequencing both DNA strands. Final sequence (accession AJ439079) was assembled by comparison with cDNA sequences (Z83835) and by sequencing polymerase chain reaction (PCR) products across subclone junctions to determine orientation.

Plant transformation and selection

Cosmid pNCED1.1 was propagated in E. coli DH5α, and then introduced by electroporation into Agrobacterium tumefaciens LBA4404. Agrobacterium-mediated transformation of Lycopersicon esculentum Mill. cv. Ailsa Craig (wild type) was performed as previously described (Bird et al. 1988). Primary transformed plants (T₀) were transferred to a partially environmentally controlled greenhouse, grown to fruiting in a peat-based compost (Levington M2; Levington Horticulture, Bamford, Suffolk, UK; in 22 cm pots) and T₁ seed extracted. Day/night temperatures were maintained at approximately 22/18 °C by computer-controlled shading, ventilation and heating. Natural illumination was supplemented for 16 h per day to give a minimum irradiance of 150 μmol m⁻² s⁻¹ (photosynthetically active radiation) at crop height, and plants were watered daily by hand. T₀ plants and then T₁ families were grown together with wild-type and notabilis control plants in the glasshouse and inspected at least twice a week under weather conditions ranging from overcast to bright sunlight. Growth habit and tendency to wilt were visually scored relative to wild-type and notabilis plants.

Southern blotting

Genomic DNA was prepared from leaves and analysed by Southern blotting as previously described (Burbidge et al. 1999). Blots were hybridized to a random primed DNA probe consisting of the complete open reading frame (ORF) of LeNCED1. Blots were washed at high stringency: 65 °C in 0.1% sodium dodecyl sulphate and 0.1× SSC (sodium chloride–sodium citrate buffer).

Growth conditions for evaluation of phenotype

Seeds of wild type, notcomp13, notcomp1 and not were imbibed for 1 h in deionized water, and then sown in 7-cm-diameter, 290 cm³ plastic pots, 2 to 4 seeds per pot. The pots were filled with a 2 : 1 (v/v) mixture of Green Formula Growing Mix (Lambert Peat Moss, Inc., Riviere Ouelle, Quebec, Canada) and sand. Scott’s Osmocote Plus 15-9-12 (Scotts-Sierra Horticultural Products Company, Marysville, OH, USA) was incorporated into the soil mixture at a rate of 7 g L⁻¹ (according to the high application rate recommended by the manufacturer) to provide sufficient micro- and macronutrients. The pots were placed in controlled environment chambers with a day length of 14 h, day/night temperatures of 25/20 °C, and a photosynthetic photon flux density of 600 μmol photons m⁻² s⁻¹ at 48 cm above the base of the pots (supplied by incandescent and cool-white fluorescent lamps). At 2 to 4 d after emergence (DAE), individual seedlings were transplanted into 17 cm diameter, 2500 cm³ plastic pots containing the same soil mixture. Plants were tracked individually according to date of emergence.

Well-watered and controlled water stress treatments

Well-watered plants of all genotypes were grown at 80% (day and night) relative humidity, and were harvested for leaf ethylene evolution rate at 17 DAE, and for shoot and root growth and leaf ABA content at 25 DAE. No attempt was made to compensate for differences in shoot and root status between the genotypes because well-watered leaf water potential was a parameter for evaluating complementation. In contrast, to allow a meaningful comparison of the effect of complementation on water stress-induced ABA
accumulation, it was essential to compare genotypes at the same level of stress, namely at the same leaf water potential and also degree of soil drying. Achieving equivalent soil drying as well as leaf water potentials was important because of the likely contribution of root-sourced ABA to the increased levels of ABA in leaves of plants in drying soil. This strategy required that the relative humidity regimes varied for the different genotypes as necessary to compensate for differences in transpiration rate after withholding water from the soil. The differences in transpiration rate were due both to the smaller size of not and because of differences in stomatal behaviour between the genotypes.

To impose controlled water stress, water was withheld at 9 DAE. The rate of soil drying was monitored by daily measurements of pot weight loss (from a uniform starting weight) and midday leaf water potential. The leaf water potential began to decline below well-watered values at approximately 17 DAE, and was then held at approximately −0.7 MPa until day 25 for all genotypes by weighing the pots three times a day at the same times each day, and adding water (via trays in which the pots were standing) as necessary to maintain constant pot weights. To achieve equal leaf water potentials between the water-stressed plants of the different genotypes, *not* remained in 80% day/night relative humidity throughout the experiment, whereas at 15 DAE the humidity regime for the wild type and *not* was changed to 70% day/night, and for *not* to 85% during the day and 99% at night (the highest the growth chambers could achieve). In spite of the high humidity, pot weights of *not* had to be held slightly higher than for the other genotypes in order to achieve equivalent leaf water potentials.

Leaf water potentials were measured by isopiestic thermocouple psychrometry (Boyer & Knipling 1965) on approximately 1 cm² sections excised from mature leaves.

**Leaf ethylene evolution**

The ethylene evolution rate of leaflets excised from the youngest three main stem leaves of well-watered plants was measured 17 DAE, as previously described (Sharp et al. 2000). The measurements were made before the induction of wound-induced ethylene, and are considered to be good estimates of the rate of ethylene evolution by the leaves of intact plants (Jackson & Campbell 1976).

**Growth evaluation**

Well-watered plants were harvested at 25 DAE. Total leaf area was measured using a leaf area meter (Model LI-3100; Li-Cor Inc., Lincoln, NE, USA). Shoot fresh weight and dry weight and root dry weight were measured gravimetrically. Because of the potential for wound-induced ethylene induction that might have affected shoot growth and development, as well as the slight reduction in total leaf area due to sampling, separate well-watered plants were used for growth, water potential and ethylene measurements.

Leaf ABA content of well-watered and controlled water stress treatments

At 25 DAE, leaflets from the three youngest main stem leaves of well-watered plants and plants experiencing controlled water stress were sampled for ABA immediately after the same plants were sampled for leaf water potential measurements. Leaf samples were immediately frozen in liquid nitrogen, freeze-dried, finely ground, and extracted in deionized water at 4 °C for 16–20 h. ABA content was measured in duplicate for each sample by radioimmunoassay using monoclonal antibody AFRC MAC 252 (Quarrie et al. 1988). This assay was validated for tomato leaves by parallel gas chromatography (GC)-mass spectrometry (Mulholland 1994).

**Leaf ABA content of rapidly dehydrated leaves**

Leaflets of the second youngest main-stem leaf were detached from well-watered plants during the light period at 24 to 27 DAE. The fresh weight of each leaflet was measured, and then leaflets were floated on double-deionized water at room temperature for 1 h to reach full saturation. (A preliminary time course determined that 1 h was an adequate length of time to reach saturation.) The leaflets were then gently blotted to remove excess water, weighed, allowed to bench dry to 76% of their saturated weight, and then incubated in air-tight plastic bags with moist filter paper for 5 h at room temperature on the bench. The leaflets were re-weighed and immediately frozen in liquid nitrogen before storage at −70 °C. They were then assayed for ABA by radioimmunoassay.

The experiment was repeated for wild type and *not* using a faster rate of drying (achieved by periodically blowing air over the leaves) that was equivalent to the drying rate of *not* in the first experiment.

**Statistical analysis**

Analyses of variance were performed with means compared using Fisher’s least significant difference test at the *P* = 0.05 level.

**RESULTS**

**LeNCED1 promoter sequence**

A tomato genomic cosmid library (Dixon et al. 1996) constructed in a vector suitable for *Agrobacterium*-mediated plant transformation was screened using PCR primers specific for the coding region of *LeNCED1*. A positive clone was fully sequenced (accession AJ439079) and named pNCED1.1 (Fig. 1).

pNCED1.1 has an insert size of 19,018 bp, and the *LeNCED1* full-length cDNA (Burbridge et al. 1997; accession Z97215) aligns with region 5404–5750 bp. The putative transcription start site is therefore at bp 5404. No introns were detected within the region of the cDNA. No other significant ORFs are present within the cosmid insert.
The 5.4 kb upstream sequence was searched for plant cis-acting regulatory DNA elements using the PLACE database (Higo et al. 1999) and several noteworthy elements were identified (Table 1). The LeNCED1 ORF has an A + T content of 41.4%. In contrast, the 5.4 kb upstream sequence has an overall A + T content of 74%, including a region from -595 to -3301 bp (relative to the putative transcription start) with 77.8% A + T that is uninterrupted by any significant G + C rich regions. The 90% AT box, a 20-bp box of which at least 18 bases are A or T (Michalowski et al. 1999), occurs at high density throughout the A + T-rich region (Fig. 1).

The 19 kb genomic clone contains a number of repeat regions of note (Fig. 1, features a–f). Two elements with 82–95% homology to TAPIRs (tomato anionic peroxidase inverted repeats) (Hong & Tucker 1998; Mao et al. 2001) are present, one either side of the ORF. A BLAST search of GenBank indicated that TAPIR elements are common within intragenic DNA of the Solanaceae, being present within at least 16 loci from Lycopersicon and three from Solanum species. Also upstream of the ORF is a unique 695 bp region of direct repeats. Downstream of the second TAPIR is a stowaway-like element (Bureau, Ronald & Wessler 1996) with 82–94% homology to at least three other tomato genomic sequences (Z54199, AF411807, M83314) and several tomato and potato ESTs. Next is a GC-rich element with homology to an insertion into the tomato phytoene synthase gene that caused the yellow flesh mutation (Fray & Grierson 1993), and homology to elements within three additional tomato genomic sequences (M88487, AF275345, AF411809) and several tomato ESTs. Finally there is a region of 220 bp (Fig. 1, feature f) with homology to a region upstream of a gene encoding a putative centromere protein (AF275345).

Complementation of not

To provide additional evidence that the not mutation is caused by the single A/T base pair deletion in LeNCED1 (Burbidge et al. 1999), the 19 kb wild-type tomato genomic

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### Table 1. Sequence motifs within the LeNCED1 promoter region

<table>
<thead>
<tr>
<th>Sequence motif</th>
<th>Position</th>
<th>Potential role (consensus sequence)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CATATG</td>
<td>-421</td>
<td>Auxin response</td>
<td>(Xu, Hagen &amp; Guilfoyle 1997)</td>
</tr>
<tr>
<td>AATTCAATTAATC</td>
<td>-365</td>
<td>Ethylene response (AWTTAAA)</td>
<td>(Montgomery et al. 1993)</td>
</tr>
<tr>
<td>CACATG</td>
<td>-244</td>
<td>ABA, dehydration</td>
<td>(Abe et al. 1997)</td>
</tr>
<tr>
<td>CCAACACGTG</td>
<td>-161</td>
<td>G-box (CACGTG)</td>
<td>(Menkens, Schindler &amp; Cashmore 1995)</td>
</tr>
<tr>
<td>GATAAA</td>
<td>19 sites</td>
<td>I-box, Light response</td>
<td>(Terzaghi &amp; Cashmore 1995)</td>
</tr>
<tr>
<td>TAACGTG</td>
<td>-1792</td>
<td>Dehydration</td>
<td>(Urao et al. 1993)</td>
</tr>
</tbody>
</table>

Previous described sequence motifs were identified using the PLACE database (Higo et al. 1999). All positions are given relative to the putative LeNCED1 transcription start.
fragment carried in a cosmid T-DNA vector (pNCED1.1, Fig. 1) was transformed into tomato plants homozygous for the not mutant allele. Of 26 kanamycin resistant lines produced, 11 were putatively complemented as they apparently had reduced epinasty, improved growth and reduced tendency to wilt when compared to control plants in the glasshouse. All 11 lines contained the wild-type LeNCED1 transgene as determined by Southern blotting using TspRI/ SalI and XhoI restriction enzyme digests (data not shown, but see Figs 2 & 3 for progeny analysis). Two lines, not-comp.1 and not-comp.13, were selected for further analysis because they were the most similar to the wild type in appearance and wilting behaviour.

Molecular analyses were performed to confirm the genotype of not-comp.1 and not-comp.13. A TspRI/SalI restriction fragment length polymorphism was used to distinguish wild-type LeNCED1 from the mutant form of this gene present in not, which lacks a TspRI site due to the single base pair deletion (Burbidge et al. 1999). Southern blot analysis was performed on T1 families produced by selfing of the not-comp.1 and not-comp.13 T0 plants. In the case of not-comp.13 15 T1 plants were scored successfully and all were found to contain the deletion (loss of TspRI site resulting in a 2.4-kb band, Fig. 2), confirming that the parent plant was homozygous not. In addition, 10 of 15 lines contained wild-type LeNCED1 (1.0 and 1.4 kb bands, Fig. 2), consistent with a 3:1 segregation of the T-DNA. Similar results were obtained for not-comp.1 of 24 T1 plants all had the 2.4 kb band and 19 had the 1.0 and 1.4 kb bands (data not shown). Independent T-DNA integrations were confirmed with XhoI, which produced a DNA fragment spanning the integration at the left border (Fig. 1). The LeNCED1 coding region probe hybridized to one band in the wild type, as expected (Burbidge et al. 1999), not-comp.1 had one additional XhoI band, and not-comp.13 had two additional bands (Fig. 3). In the case of not-comp.13, no segregation between the two XhoI T-DNA bands was observed in 18 T1 lines, consistent with close linkage between them (data not shown). All not-comp.1 and not-comp.13 T1 lines were visually scored for reduced tendency to wilt in the glasshouse, and in all cases this trait co-segregated with the T-DNAs.

This molecular analysis established that the not-comp.1 and not-comp.13 lines have the genotype required for complementation studies. For both not-comp.1 and not-comp.13, homozygous lines were produced for both not and the LeNCED1 T-DNA by selecting T1 generation plants whose progeny were all kanamycin resistant. These double homozygote, putative complemented lines were used for subsequent physiological analysis.

Whole plant physiological analysis of not-comp.13 and not-comp.1

Well-watered growth and ethylene production

In comparison with wild type, not showed greatly reduced shoot and root development (Figs 4 & 5). Shoot and root dry weights were 54 and 52% of the wild type, respectively, and shoot fresh weight and total leaf area were also significantly reduced. Stem length of not was also reduced, but not significantly (not shown). Marked leaf epinasty (Fig. 4a) and prolific adventitious rooting (Fig. 6) occurred in not but were absent in the wild type.

Shoot and root growth of not-comp.13 and not-comp.1 were greatly improved in comparison with not. For all parameters measured, the transgenic lines were not significantly different from the wild type, although the growth of not-comp.1 was slightly reduced, having 86 and 85% of the root and

Figure 2. Southern blot analysis showing segregation of mutant and wild-type alleles of the not gene in line not-comp.13. Genomic DNA from 15 T1 not-comp.13 plants was digested with TspRI and SalI and the LeNCED1 ORF was used as the probe. Control DNA from not and wild type (WT) was included. Band sizes in kilobase pairs (kb) are indicated.

Figure 3. Southern blot analysis of genomic DNA from not-comp.13 (13), not-comp.1 (1) and not (N). DNA was digested with XhoI, and the ORF from LeNCED1 was used as a probe. Size markers in kb are indicated.
shoot dry weights, respectively (Fig. 5). The morphologies of the shoot (Fig. 4a) and root (Fig. 4b) were very similar to the wild type in both the transgenic lines. Adventitious rooting was absent in not_{comp.13}, and was either non-existent or very sparse in not_{comp.1} (Fig. 6). So, with respect to growth, not_{comp.13} appeared to be fully complemented by the transgene. However, for not_{comp.1}, there was some evidence that complementation was incomplete.

Leaves of well-watered plants of both transgenic lines evolved ethylene at the same rate as the wild type, whereas not evolved ethylene at twice the rate (Fig. 7). These ethylene data are consistent with the observation that of the four

Figure 4. Appearance of the shoot (a) and root system (b) of representative plants of well-watered wild type (WT), not_{comp.13}, not_{comp.1}, and not at 25 DAE. Plants of not exhibited severely stunted shoot and root development and leaf epinasty.
Complementation of *notabilis* genotypes only *not* showed symptoms of excess ethylene such as epinasty and prolific adventitious rooting (Figs 4 & 6). A similar increase in leaf ethylene evolution was reported for well-watered plants of *flacca* cv. Rheinlands Ruhm (Sharp et al. 2000).

**Leaf ABA content under well-watered and controlled water stress conditions**

Under well-watered conditions *not* had a significantly lower leaf water potential than the wild type (−0.57 MPa compared with −0.44 MPa), and *not*comp.1 and *not*comp.13 were intermediate between these values (Fig. 8). Bulk leaf ABA content in well-watered plants was not significantly different between wild type, *not*comp.13 and *not*comp.1, but was significantly reduced to 53% of wild type in *not*.

When all four lines were mildly water-stressed such that their leaf water potentials were held at the same value (approximately −0.7 MPa) for several days, wild type and *not*comp.13 showed a similar response with ABA content increasing significantly by 101 and 118% above the respective well-watered values (Fig. 8). In *not*comp.1, although the basal level of ABA was at the wild-type level, the response of ABA content to stress was much less than in wild type and *not*comp.13, with only a 41% increase above the well-watered value. The ABA content of *not*comp.1 under stress was not significantly greater than in well-watered wild type or *not*comp.1, and was significantly less than in stressed wild type or *not*comp.13. In *not*, the increase in ABA content under stress was also insignificant. These data indicate again that *not*comp.13 appeared to be fully complemented and that *not*comp.1 was only partially complemented.

**ABA content of rapidly dehydrated leaves**

ABA accumulation in rapidly dehydrated detached leaves was determined for the four genotypes (Fig. 9). In comparison with the ‘basal’ ABA content of leaves from well-watered plants (Fig. 8), rapidly dehydrated wild-type leaves showed a massive accumulation (from 150 to 3000 ng g⁻¹ FW). This response falls within the range of previous reports for dehydrated, wild-type tomato leaves in which ABA accumulated to a level ranging from 1000 ng g⁻¹ FW (Neill & Horgan 1985) to 1600 ng g⁻¹ FW (Cohen & Bray 1990) to 3500 ng g⁻¹ FW (Kahn et al. 1993). Our data is at the higher end of this range, possibly reflecting the extent of dehydration (dried to 76% initial FW here compared with 88–90% in the three cited examples), or alternatively it might reflect the physiological or biochemical status of the tissue at harvest, as determined by growth conditions. In the case of *not* there was virtually no accumulation of ABA in response to dehydration (Fig. 9). Neither *not*comp.1 nor *not*comp.13 were fully complemented under this extreme water-deficit stress; both lines accumulated only one-third of wild-type levels of ABA.

In this experiment the leaflets of the various genotypes did not all dry at the same rate prior to the 5 h incubation. From most to least rapid, the rank order of drying rate was as predicted by degree of complementation and ABA content in whole plants (Fig. 8) and therefore the expected ability for stress-induced stomatal closure: *not > not*comp.1 > *not*comp.13 = wild type. The leaflets of *not* and *not*comp.1 reached 76% of the saturated fresh weight 1–2 h faster than the approximately 3.5 h required for the wild type and *not*comp.13. Because the drying times were of a different duration, and with the genotype drying the quickest...
When the drying rate of wild type and notcomp.13 was increased to within the 1–2 h range of notcomp.1 and not, the increase in leaf ABA (Table 2) was very similar to that of the first assay (Fig. 9). This indicates that the majority of the ABA accumulation occurred during the 5 h incubation period and not during the drying period. As in the first assay, the wild type produced three-fold more ABA than notcomp.13. Leaf water potentials of wild type and notcomp.13 were equivalent, so differences in leaf water status could be ruled out as a cause of the different ABA accumulation in the leaflets (Table 2). These data indicate that even notcomp.13 was not fully complemented.

DISCUSSION

Transformation of tomato not homozygotes with a large (19 kb) genomic fragment containing the LeNCED1 gene has shown that the mutant could be complemented to varying degrees. Two independent complementation lines, notcomp.13 and notcomp.1, were studied in detail to provide strong evidence that the shared complementation phenotypes were due to the T-DNA rather than due to perturbation of a host gene at the integration site.

In whole plants, there were no significant differences between wild type and notcomp.13 for all measurements of growth, water relations, leaf ABA content and leaf ethylene evolution in well-watered plants, nor for increase in leaf ABA content under mild water stress. In contrast, notcomp.1 was intermediate between wild type and not for some of
These parameters, most notably the ability for stress-induced ABA accumulation, and was thus apparently only partially complemented. However, it became clear that even not\textsubscript{comp.13} was unable to respond to an extreme stress treatment, namely rapid dehydration of detached leaves, as effectively as the wild type. The not\textsubscript{comp.13} line could not match the 20-fold increase in ABA levels produced by the wild type.

Both of these partially complemented transgenic lines will be useful in determining the physiological roles of stress-induced ABA increases since they are the mildest ABA-deficient genotypes available in tomato, and have essentially normal basal ABA concentrations and shoot and root development under well-watered conditions. All the existing tomato ABA-deficient mutants have non-stressed ABA levels that are low in comparison with wild type (Neill & Horgan 1985; Jones, Sharp & Higgs 1987; Cornish & Zeevaart 1988; Parry, Griffiths & Horgan 1992; Holbrook \textit{et al.} 2002). This ‘basal deficiency’ prevents normal water relations, growth and development of these plants even under optimum conditions (Sharp \textit{et al.} 2000).

We are now using the partially complemented transgenic lines in which non-stressed ABA levels are apparently normal, but stress-induced ABA accumulation is restricted, to investigate the involvement of stress-induced ABA in plant responses to water stress. The not\textsubscript{comp.1} transgenic plants provide a novel genotype to investigate the involvement of increased levels of ABA in controlling growth and stomatal behaviour during mild water deficits. The not\textsubscript{comp.13} transgenic plants provide a novel genotype that will allow investigation of whether or not the failure to rapidly accumulate very high levels of ABA in detached leaves has any physio-

\textbf{Figure 8.} Midday leaf water potentials (top two graphs) and leaf ABA content (bottom two graphs) of wild type (WT), not\textsubscript{comp.13} (13), not\textsubscript{comp.1} (1), and not (not) under well-watered conditions (left panels) or during controlled water stress (see Methods; right panels). For well-watered plants, leaf water potentials are averages of values from 19 to 25 DAE. For water-stressed plants, leaf water potentials are averages of measurements from 22 to 25 DAE during the period of controlled water stress. Leaf ABA contents were measured at 25 DAE on the same plants used for water potential measurements. Data are means ± standard error (leaf water potentials, \textit{n} = 8–16; ABA contents, \textit{n} = 3–6). For combined well-watered and water-stressed treatments, different letters show values that are significantly different at the 0.05 level. FW, fresh weight.
Leaflets were detached from well-watered wild type (WT), \textit{not}_{comp.13} (13), \textit{not}_{comp.1} (1) and \textit{not} (not) at 25 DAE, allowed to dry to 76\% of their saturated fresh weight, and incubated for 5 h before measurement of ABA content. The drying times varied from 1.5 to 3.5 h depending on the genotype (see Results). Data are means ± standard error (\( n = 4–5 \)). Different letters above the bars show values that are significantly different at the 0.05 level. FW, fresh weight.

One explanation for the difference between the two lines is that T-DNA copy number in \textit{not}_{comp.13} and \textit{not}_{comp.1} was two and one, respectively. If the genomic clone lacked a long-range element that controls the level of transcription, increasing transgene copy number could compensate for this defect. Alternatively, it is possible that all the necessary regulatory elements are present in \textit{pNCED1.1} but partial complementation results from the influence of host flanking sequences, or from the partial co-suppression of an otherwise fully active transgene by the endogenous \textit{not} gene. One trivial explanation, that of incomplete transfer of the 5.4 kb promoter region, is unlikely as it lies between the \textit{LeNCED1} and \textit{nptII} ORFs which are both functionally active in the transgenic plants. Whatever the mechanistic explanation, as \textit{LeNCED1} is an environmentally responsive gene encoding a rate-limiting enzyme, the ability to recreate a perfect wild-type phenotype is likely to depend on the precise level and behaviour of transgene expression, rather than a threshold level of active gene product.

Attempts to measure \textit{LeNCED1} mRNA in the complemented lines are hampered because of the high background levels of \textit{LeNCED1} mRNA in \textit{not}. Although the \textit{not} allele produces a truncated protein, \textit{LeNCED1} mRNA level is the same as in the wild type (unpublished results). A fully complemented line would contain mRNA transcribed from both the endogenous \textit{not} gene and the transgene and might therefore be expected to have double the \textit{LeNCED1} mRNA compared with \textit{not}. A partially complemented line might have more \textit{LeNCED} mRNA than \textit{not}, but less than the two-fold increase expected of a fully complemented line. In addition \textit{LeNCED1} mRNA is highly variable depending on leaf water status (Thompson et al. 2000a). Distinguishing expression levels between different complemented lines would therefore require a highly precise quantification that would be difficult to achieve by northern or reverse transcriptase-PCR analysis.

An insight into the requirements for native \textit{LeNCED1} gene expression may be gained by analysing the genomic regions flanking the \textit{not} gene. A 3 kb region of AT-rich sequence (77.8\%) begins 595 bp upstream of the transcription start and such AT-rich sequences are often associated with matrix attachment regions (MARs) and origins of DNA replication (Boulakis 1995). In a study of randomly obtained MARs from tobacco, the 90\% AT box was identified as the sequence element most highly correlated with

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**Table 2.** Water relations and ABA content of rapidly dehydrated leaflets

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>\textit{not}_{comp.13}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drying time to reach 76% of saturated weight (h)</td>
<td>1.6 ± 0.2</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>Percentage of saturated weight at end of 5 h incubation</td>
<td>69.7 ± 2.0</td>
<td>70.1 ± 1.7</td>
</tr>
<tr>
<td>Leaf water potential at end of 5 h incubation (MPa)</td>
<td>−1.20 ± 0.09</td>
<td>−1.11 ± 0.02</td>
</tr>
<tr>
<td>Leaf ABA content (ng g⁻¹ FW)</td>
<td>3491 ± 158</td>
<td>1160 ± 44</td>
</tr>
</tbody>
</table>

Leaflets were detached from well-watered wild type (WT) and \textit{not}_{comp.13} plants at 24–27 DAE, dried to 76\% of their saturated weight, and incubated for 5 h before measurement of ABA content. Drying times were similar to those for \textit{not}_{comp.1} and \textit{not} in Fig. 9. Data are means ± standard error (\( n = 3 \) for water potentials, and \( n = 9 \) for other data). Only the ABA content values are significantly different at \( P = 0.05 \). FW, fresh weight.

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**Figure 9.** ABA content of rapidly dehydrated leaflets. Leaflets were detached from well-watered wild type (WT), \textit{not}_{comp.13} (13), \textit{not}_{comp.1} (1) and \textit{not} (not) at 25 DAE, allowed to dry to 76\% of their saturated fresh weight, and incubated for 5 h before measurement of ABA content. The drying times varied from 1.5 to 3.5 h depending on the genotype (see Results). Data are means ± standard error (\( n = 4–5 \)). Different letters above the bars show values that are significantly different at the 0.05 level. FW, fresh weight.
MAR binding strength (Michalowski et al. 1999) and this is present at high density in the 3 kb AT-rich sequence (Fig. 1). MARs are believed to delimit chromatin domains accessible to trans-acting factors and are proposed to be involved in transcriptional regulation of native genes (Gasser et al. 1989). Further, MARs may act to reduce the variability of expression of randomly integrated transgenes by imposing a chromatin loop domain structure on the integration site thus reducing the influence of host regulatory sequences close to the T-DNA borders (Liu & Tabe 1998; Vain et al. 1999; Allen, Spiker & Thompson 2000). If the 5.4 kb of sequence upstream of LeNCED1 does contain a genuine MAR, this may help the transcriptional regulation of LeNCED1 genes within the pNCED1.1 T-DNA to be less deviant from the behaviour of the native gene but may not be sufficient to fully mimic its expression pattern. A MAR downstream of the LeNCED1 transgene may be required to define the other anchor of any chromatin loop domain. The downstream region also contains several long AT-rich stretches so it is conceivable that the 19 kb genomic clone includes a full loop domain. Appropriate chromatin organization in an extended region flanking the native not gene may be required to facilitate rapid and sustained maximal responses to acute dehydration and the ideal DNA sequence context may not have been fully recreated upon integration of the transgene.

Analysis of the promoter from 0 to –595 bp, excluding the upstream AT-rich region, reveals several putative transcription factor-binding sites relevant to the known expression patterns of the LeNCED1 gene (Table 1). Five ethylene-responsive elements upstream of LeNCED1, one of which is in the 0 to –595 bp region, are of particular note because of the proposal that ethylene stimulates NCED activity (Hansen & Grossmann 2000; Grossmann & Hansen 2001). In addition, the presence of the Arabidopsis dehydrogenase response element CACATG (Abe et al. 1997) in the LeNCED1 promoter is noteworthy as such an element could function in water-stress-induced ABA biosynthesis.

Promoters linked to reporter genes such as luciferase, GFP and GUS are often used to describe the expression pattern of promoters and to determine the contribution of individual promoter elements. However, this approach does not provide information about the physiological consequences of directing expression of a native gene product to a particular tissue, or during a specific developmental phase or environmental response. An alternative and elegant approach is suggested by the work reported here on the not gene, namely the linking of promoters, or promoter derivatives (e.g. truncations, deletions, duplications) to their native ORFs and the use of such constructs for complementation of null mutants. This would allow the complementation phenotype to become the ‘reporter’. Such an approach may allow physiological functions of promoter elements to be determined directly, rather than inferred from the pattern of reporter activity.

Complementation is a robust form of evidence to confirm the function of a cloned gene. We have achieved full complementation of not in all but one parameter, and together with mapping data and sequencing of the mutant allele (Burbridge et al. 1999) this provides convincing evidence that LeNCED1 is indeed the wild-type allele of not and has a key function in ABA biosynthesis. Our results do however, suggest that it can be difficult to demonstrate complete complementation of a null mutation in a rate-limiting enzyme, even when large regions of ORF-flanking DNA have been included in the constructs.

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REFERENCES


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