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RESEARCH PAPER

# Reduction of cell size induced by *enod40* in *Arabidopsis thaliana*

**Flavia Guzzo[1], Paolo Portaluppi[1], Romeo Grisi[1], Silvia Barone[1], Stefania Zampieri[1], Henk Franssen[2] and Marisa Levi[1,*]**

[1] *University of Verona, Dipartimento Scientifico e Tecnologico, Strada le Grazie 15, Cà Vignal 1, 37134 Verona, Italy*

[2] *Wageningen University, Department of Molecular Biology, Drijenlaan 3, 6703 HA Wageningen, The Netherlands*

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## Abstract

**An extensive analysis of organ and cell size was performed in three different *Arabidopsis* lines transformed with the early nodulin gene *enod40* under control of the *CaMV35S* promoter. All three transgenic lines presented a significant decrease in the mean size of both epidermal internode and leaf mesophyll cells. Flow cytometric and image analysis of *enod40*-transfected protoplasts prepared from wild-type *Arabidopsis* cell suspensions showed that transient expression of the gene resulted in reduced forward light scattering (a factor correlated with particle size) and cell size. The direct administration of ENOD40 peptide to fresh protoplasts also resulted in reduced forward scattering with respect to the control and to the administration of unrelated peptides. As far as is known this is the first report documenting a biological effect of *enod40* at the cellular level in non-legume plants.**

Key words: *Arabidopsis*, cell size, *enod40*, flow cytometry, GFP.

## Introduction

*enod40* was identified as a gene whose expression is highly induced after infection of legume roots with *Rhizobium* spp. The gene is induced within 3 h after root infection in the root pericycle and later on in dividing cortical cells and in the nodule primordium (Kouchi and Hata, 1993; Yang *et al.*, 1993; Compaan *et al.*, 2001). As the expression in the pericycle precedes cortical cell division, the early expression of e*nod40* suggests that this gene is involved in *Rhizobium* signal-mediated induction of cell division. Introduction of *enod40* by ballistic targeting has been shown to induce cortical cell divisions in a non-autonomous-cell manner (Charon *et al.*, 1997). Over-expression of *enod40* in *Medicago truncatula* leads to changes in the dynamics of nodulation, but not to the formation of more nodules, indicating that expression of *enod40* does not lead to cell division *per se* (Charon *et al.*, 1999). *enod40* expression has also been localized in non-symbiotic meristems of legume plants, including apices of lateral roots, young leaves, and stipule primordia (Papadopoulou *et al.*, 1996; Corich *et al.*, 1998), procambial cells (Corich *et al.*, 1998), and embryonic tissues (Flemetakis *et al.*, 2000).

*enod40* has also been cloned from non-legumes, such as tobacco (van de Sande *et al.*, 1996), rice (Kouchi *et al.*, 1999), and more recently from tomato (Vleghels *et al.*, 2003), maize (Compaan *et al.*, 2003), *Lolium perenne*, and *Hordeum vulgare* (Knud, 2004).

The *enod40* genes isolated so far display relatively low overall sequence homology. The homology among *enod40* genes is restricted to two highly conserved regions (box I and box II). *enod40* mRNA lacks a long open reading frame (ORF), but instead several short ORFs are present. This observation has led to the hypothesis that either peptides encoded by the short ORFs are biologically active molecules (van de Sande *et al.*, 1996; Kouchi *et al.*, 1999; Compaan *et al.*, 2001; Sousa *et al.*, 2001; Rohrig *et al.*, 2002; Knud, 2004) or that *enod40* mRNA itself possesses biological activity (Crespi *et al.*, 1994; Sousa *et al.*, 2001).

Within all *enod40* genes identified to date, the 5′ conserved sequence (box I) comprises an ORF encoding for a putative small peptide that is from 10 to 13 amino acids long depending on the species (Kouchi *et al.*, 1999; Knud, 2004). This suggests that the peptide encoded within this ORF might be translated and that it represents the molecule that mediates the biological activity of *enod40*.

* To whom correspondence should be addressed. Fax: +39 45 8027929. E-mail: marisa.levi@univr.it

Although the peptide has not been detected *in vivo*, which may be due to either a high level of intrinsic instability or the rapid masking of epitopes, the presence of an antigen related to the peptide in nodule extracts of *Medicago sativa* (Sousa *et al.*, 2001) and soybean (van de Sande *et al.*, 1996) has been detected in immunocompetition experiments, indicating that the peptide might be present in tissues that display a high expression of the gene.

The expression of *enod40* in non-symbiotic tissues and its presence in non-legume plants suggest that the gene is involved not only in symbiosis, but also that *enod40* has a role in a process shared by all cell types in which the gene is expressed. Studies aimed at elucidating the role of *enod40* in plant development and at finding out whether the peptide encoded within box I is biologically active would undoubtedly benefit from the availability of mutants in *enod40*. So far only a Mu-transposon *enod40*-tagged line has been described in maize (Compaan *et al.*, 2003). However, no phenotypic growth aberration was observed, which could be explained by either the presence of a second *enod40* gene in maize or by the mild phenotype. Over-expression of *enod40* in tobacco leads to plants with adventitious shoots, while over-expression in *Medicago* interferes with nodulation (Charon *et al.*, 1999). Apparently, plants are more responsive to over-expression of *enod40* than its knock-out. To investigate further the role of *enod40* in plant development, the effect of over-expression of *enod40* was studied in the model plant *Arabidopsis thaliana*.

In this report, it is shown that *enod40* over-expression affects cell size in selected tissues. This activity could be mimicked by transient over-expression of *enod40* in protoplasts of *A. thaliana* or by administration to protoplasts of ENOD40 synthetic peptides from either soybean or tobacco.

## Materials and methods

### Plant material

Seeds of *Arabidopsis thaliana* L., ecotype Wassilewskjia, were planted in a soil/agriperlite mixture, vernalized for 3 d, and then transferred to a greenhouse with a 14 h photoperiod. For root analysis, seeds were sterilized and spread on solid MS medium in Magenta vessels, vernalized, and then transferred to a growth chamber at 25 °C with a 16 h photoperiod.

*Arabidopsis thaliana* L. plants, ecotype Wassilewskjia, carrying *CaMV35S::Gmenod40* (Van de Sande *et al.*, 1996), were generated by vacuum infiltration (Bechtold *et al.*, 1993). Seeds were selected on MS plates (Murashige and Skoog, 1962) containing 20 μg $ml^{-1}$ methotrexate. Plants resistant to the selective antibiotic were propagated and the progeny was selected twice. Plants homozygous for the presence of the methotrexate resistance gene were further analysed for the presence of *Gmenod40* DNA by Southern analysis. Expression of the transgene was confirmed by RT-PCR using *Gmenod40* specific primers (Van de Sande *et al.*, 1996). The progeny of three plants of $F_3$, lines 1.1, 3.1, and 4.4, homogenous for the presence of the methotrexate resistance gene and *CaMV35S::GmENOD40*, with the higher *enod40* expression, was used for further analyses together with the wild-type line.

The *Arabidopsis* suspension culture E157, generated from *A. thaliana* Columbia embryos, kindly provided by Professor Fiorella Lo Schiavo (Dipartimento di Biologia, University of Padova), was maintained in Gamborg's medium (Gamborg *et al.*, 1968) supplemented with 2% sucrose, on a rotary shaker at 25° and subcultured every 7 d.

### Organ and cell analysis

Internode length was measured with a graduated ruler. Leaves were photographed and measured as described below. Root diameter was measured on transverse sections. Fully expanded leaves and 5-mm-long segments of root tips from 40-d-old plants were fixed, dehydrated and embedded in paraplast (leaves) and in LR White resin (roots) by routine methods. Transverse leaf sections (7 μm) and transverse and longitudinal root sections (1 μm) were cut and stained with 0.1% Calcofluor (Fluorescent Brightener 28; Sigma) in water. The sections were observed with a fluorescence inverted Olympus IX70 microscope and images were acquired with a JVC KI-F58 CCD camera. Images were also collected, as described above, from fresh epidermis peelings of fully expanded first caulinar internode, and mature pollen grains.

Cell size and shape were analysed using the software 'Image-Pro Plus' (Media Cybernetics, LP), with manual, semi-automatic, or fully automatic measuring options. Analysis of variance of the data was performed with Microsoft Excel. At least 300 cells from at least 10 different plants were measured for each sample.

### Preparation of plasmids for transient expression

For the transient expression assay, a construct (termed 'double construct') allowing the simultaneous expression of a reporter gene and *enod40* was prepared and inserted in pGEM 3Zf(+). The construct contained a *gfp* (green fluorescent protein) variant reporter gene under *CaMV35S* promoter and an expression box X (containing *CaMV35S*, a small polylinker and *nos* terminator), in which the coding sequence of the gene of interest can be inserted. Soluble modified (sm) variants of GFP were used. The pUC118 clone of *smrs* (red shift)-*gfp* was obtained from ABRC (Arabidopsis Biological Resource Centre, Ohio State University, USA). A 442 bp fragment of *Gmenod40*-2 (accession number X86442) containing the ORF of the peptide cloned in pMON999 was used.

Intermediate plasmids were prepared by insertion of *smrs-gfp*, *CaMV35S-nos,* and *enod40* sequences into pBluescript SK(+). Sequences excised from intermediate vectors and pGEM 3Zf(+) were used for the preparation of the following plasmids for protoplast transformation: pRSGFPX (with the expression box X empty) and pRSGFPXENOD (with the expression box X containing the *enod40* sequence).

### Protoplasts preparation and transformation

Ten-day-old seedlings of control and transformed plants were cut into pieces and incubated with 1% cellulase R10 and 0.25% macerozyme R10 (Yakult Pharmaceuticals) in 10 mM 2-(*N*-morpholino)ethane sulphonic acid (MES) buffer containing 30 mM $CaCl_2$ and 0.5 M mannitol for 3–4 h with gentle agitation at room temperature. The suspension was then filtered through a 50 μm mesh filter, washed twice with 0.5 M mannitol and resuspended at a concentration of 200 000 protoplasts $ml^{-1}$ in Gamborg's medium containing 2% sucrose, 0.5 M mannitol, 0.5 mg $l^{-1}$ 2,4-D (2,4-dichlorophenoxyacetic acid), and 0.25 mg $l^{-1}$ 6-BAP (6-benzylaminopurine). Protoplasts were analysed by flow cytometry and image analysis (see below).

For transient expression experiments, protoplasts were prepared from a suspension culture of the E-157 cell line, incubating cells (3 d post-inoculation) with enzymatic solution (2% cellulase, 1% macerozyme, and 0.5 M mannitol in 50 mM Na-citrate buffer, pH 4.8) overnight in the dark at 25 °C with gentle agitation. Thereafter, the

suspension was treated as in Abel and Theologis (1998) for polyethylene glycol (PEG)-mediated transformation. Briefly, the suspension was filtered through a 50 μm mesh filter, washed in $CaCl_2$ and mannitol and resuspended in W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM glucose, and 1.5 mM MES–KOH, pH 5.6). Plasmid DNA (20–100 μg) and an equal amount of carrier DNA were mixed, precipitated, and resuspended in 50 μl sterile water. The protoplast suspension (300 μl, corresponding to $\sim 1.5 \times 10^6$ protoplasts) was added to DNA and gently mixed; 300 μl of PEG solution (400 mM mannitol, 100 mM $Ca(NO_3)_2$, and 40% v/v PEG 3350; Sigma) were added and the mixture was incubated for 30 min at room temperature. After several washes in W5 solution, protoplasts were resuspended in 3 ml of Gamborg's medium+0.5 mg $l^{-1}$ 2,4-D and 0.5 M mannitol, and incubated for 16–24 h in Petri dishes (60 mm diameter) in the dark at 25 °C.

For the analysis of the effect of peptide administration, cell suspensions were incubated in the enzyme mixture for 8 h and then washed three times in Gamborg's medium with 2% sucrose and 0.5 M mannitol, and cultured in the same medium.

### *Peptide administration*

The following peptides were used: *Gm*ENOD40 (MELCWLTTIHGS) (a), *Nt*ENOD40 (MQWDEAIHGS) (b), unrelated peptide M1.2 (LLLLTVLTV) (c), and gramicidin S (d). Three Petri dishes for each treatment were prepared and analysed in each experiment. Experiments were performed at least four times with peptides (a) and (b), and twice with peptides (c) and (d).

### *Fluorescence microscopy*

Calcofluor-stained sections were observed with an Olympus IX70 inverted microscope, equipped with a WU filter (excitation 330–385 nm, dichroic mirror 400 nm, emission >420 nm). Protoplasts were observed with the same microscope, with the filter combination NB (excitation 470–490 nm, dichroic mirror 500 nm, emission >515 nm).

### *Flow cytometry*

Flow cytometric analysis (FCM) of protoplasts was performed with a Bryte HS (Bio-rad) cytometer, equipped with a mercury-xenon lamp. Forward-angle light scattering (FS) and large-angle light scattering (SS) include photons deflected at angles up to 2° and 15°, respectively. The following filter blocks were used: excitation block FITC 520 (excitation filter 470–490 nm; beam splitter 510 nm; emission filter >520 nm); separator block GR1 (emission filter 1, FL1, 515–565 nm; beam splitter 560 nm; emission filter 2, FL2, 590–720 nm). Fluoresbryte beads (4.5 μm diameter) were used for calibration of the instrument and as reference internal standard for light scattering detection.

Monoparametric histograms and biparametric cytograms of FS, SS, FL1, and FL2 were acquired in listmode with log amplification. For each acquisition at least 1200 cells were measured. For each sample, three different acquisitions were collected, with highly reproducible results. The experiments were performed at least three times with similar results. The figures show one representative experiment.

For the identification of transformed protoplasts, a threshold was fixed on the basis of the autofluorescence of the untransformed control. In the transformed sample, protoplasts with green fluorescence higher than the threshold were considered transformed. For the analysis of the effect of *enod40* expression, FS values of *smRS-GFP*- and *enod40*-expressing protoplasts were compared with those of a control expressing *rsGFP* only.

In peptide administration experiments, protoplasts were stained with 10 μg $ml^{-1}$ fluorescein diacetate (FDA) for 5 min at room temperature prior to FCM analysis. A threshold was fixed on the basis of the autofluorescence of the unstained control; protoplasts with green fluorescence higher than the threshold were considered viable and analysed for their FS values.

Flow cytometry data were converted in FCS (flow cytometry standard) format and analysed with WinMDI software (http://facs.scripps.edu/software.html).

## Results

### *Flow cytometric analysis of protoplasts from plants over-expressing* enod40 *revealed a reduction in cell size*

Three *enod40*-transformed *Arabidopsis* lines (1.1, 3.1, and 4.4) were chosen for their high *enod40* expression. Initially, protoplasts were isolated from 10-d-old whole plantlets and from mature leaves of wild-type (wt) and 1.1, 3.1, 4.4 *enod40*-over-expressing lines, and analysed by flow cytometry. Differences in forward light scattering (FS) were found between the protoplasts isolated from transformed lines and wt protoplasts. The mean FS values of protoplasts from transformed lines (1.1, 1260; 3.1, 1265; and 4.4, 1275 arbitrary units) were significantly lower ($P < 0.01$) than those from the wt (1337). Image analysis of the same protoplasts showed that the area of protoplasts isolated from the transgenic lines was significantly smaller ($P < 0.01$) than that of protoplasts isolated from wt plants.

### Arabidopsis *plants over-expressing* enod40 *showed reduced cell size in some tissues*

The overall appearance of transgenic plants was very similar to that of wt, with the same number of organs, phyllotaxis, and branching in vegetative and reproductive shoots. As preliminary flow cytometry experiments suggested that transgenic plants should have some cells that are reduced in size, an extensive analysis of the size of different organs and cells was performed. No significant differences were found between organ sizes in all three transgenic lines compared with wt. With regard to individual cells, in all three transgenic lines the size of epidermal cells of the first internode and of mesophyll cells was significantly lower than in the wild type; that of pollen grains and of endodermal cells was lower in two transgenic lines (Table 1).

### *Protoplasts transiently expressing* enod40 *showed reduced expansion*

As a second approach, the effect of transient expression of *enod40* in *Arabidopsis* protoplasts was examined by preparing a 'double construct' that allowed the detection of the simultaneous expression of a reporter gene and another gene of interest. *sm*(*s*oluble modified; Davis *et al.*, 1998)*rs*(red shifted)-*gfp* was used as the reporter gene.

Protoplasts were transiently transformed with pRSGFPXENOD, in which *enod40* was inserted in the expression box 'X' of pRSGFPX. RT-PCR performed on protoplasts 24 h after transformation showed the simultaneous

**Table 1.** *Area ($\mu m^2$) of selected types of cells (mean±standard error) in wt and* enod40 *transgenic lines*

The significance of differences between each transgenic line and the wt was determined: ** $P<0.01$; *** $P<0.001$.

| | No. of cells measured | wt | 1.1 | 3.1 | 4.4 |
|---|---|---|---|---|---|
| Endodermal cells | >350 | 1266.2±21.37 | 1299.9±29.70 | 1077.0±18.91*** | 1114.9±25.58*** |
| 1st internode epidermal cells | >300 | 3255.6±94.56 | 2358.5±85.65*** | 2450.3±93.20*** | 2535.5±75.62*** |
| Mesophyll cells | >800 | 578.9±20.56 | 444.9±13.82*** | 517.7±15.58** | 375.5±11.29*** |
| Pollen grains | >500 | 433.2±1.07 | 415.6±1.64*** | 423.7±1.00*** | 430.7±1.04 |

expression of the two genes, whereas in control protoplasts transfected with pRSGFPX (with an empty expression box), only smRSGFP mRNA was detected (not shown). *smrs-gfp* was chosen as the reporter gene instead of *smgfp* due to its higher excitation with blue light, which also permitted light scattering analysis.

FCM analysis of protoplasts was performed at the end of the transformation procedure and 24 h later. As shown in Table 2, the mean FS values increased in this period and those attained by protoplasts expressing *smrs-gfp* and *enod40* were significantly lower than those of control protoplasts (expressing *smrs-gfp* alone). The shoulder in the distribution of FS values of *enod40*-transfected protoplasts (Fig. 1) indicates that *enod40* expression results in a higher proportion of protoplasts with low FS, which should have a smaller size. Accordingly, image analysis made on 350 protoplasts for each sample showed that the area of *enod40*-transformed protoplasts was significantly lower than that of controls (3566 versus 4282 $\mu m^2$; $P<0.005$). This was due to a higher content in small protoplasts, as shown in Fig. 2. These differences were evident after 24 h of protoplast culture, when cell division is not yet restored in transfected protoplasts.

### *Protoplasts treated with ENOD40 peptide showed reduced FS*

To verify whether the effect of over-expression of *enod40* could be due to the peptide, protoplasts were directly treated with ENOD40 peptide. Flow cytometric analysis of FS was performed on fresh protoplasts (T0) and after 17 h of culture. To identify viable protoplasts, the suspensions were stained for 5 min with FDA prior to FCM analysis.

Initial experiments with concentrations of the peptide between $10^{-8}$ and $10^{-5}$ M showed that the maximal effect on FS values was found at a concentration of $10^{-6}$ M. Protoplasts were then treated with ENOD40 peptide and two unrelated peptides at this concentration. As shown in Table 3, the mean FS value attained by *Nt*ENOD40-treated protoplasts was significantly lower than that attained by untreated protoplasts and protoplasts treated with unrelated peptides. The effect of *Gm*ENOD40 peptide was very similar to that of the tobacco peptide (data not shown).

## Discussion

In the present report, three different systems were used to verify a possible effect of *enod40* in the non-legume model plant *Arabidopsis thaliana*: (i) three lines of *Arabidopsis* plants transformed with *enod40* under control of the *CaMV35S* promoter, obtained from independent transformation events; (ii) *Arabidopsis* protoplasts transiently expressing the *enod40* gene; (iii) *Arabidopsis* protoplasts treated with ENOD40 peptide. The results obtained in all these systems are highly concordant and demonstrate that *enod40* induces a limitation of cell size, both in intact plants and in protoplasts derived from suspension cell culture.

**Table 2.** *Mean FS values (arbitrary units) of protoplasts at the end of the transformation procedure (t0) and 24 h later (*smrs-gfp *and* smrs-gfp+enod40*)*

The difference between FS values in protoplasts transfected with *smrs-gfp* alone and those transfected with *smrs-gfp* and *enod40* was statistically significant ($P<0.001$).

| | No. of transfected protoplasts | Forward scattering (mean±SE) |
|---|---|---|
| t0 | 1308 | 981.8±3.01 |
| *smrs-gfp* | 1308 | 1191.1±4.78 |
| *smrs-gfp+enod40* | 1308 | 1160.3±4.65 |

In earlier flow cytometric studies, when isolated from whole plantlets or mature leaves, protoplasts from all three transgenic lines showed mean forward scattering levels that were significantly lower than those of protoplasts isolated from wt plants. This was due to an increase in protoplasts with lower FS values. Since FS is a parameter correlated with particle size (Kerker, 1983), these results suggested that a certain number of protoplasts from transformed plants (and hence cells from which these protoplasts had been isolated) had smaller sizes than wt. Direct size measurement of protoplasts with a microscope-based image analysis program confirmed this conclusion. FS can thus be considered a good marker of protoplast size in the present system. Nonetheless, the location of the cells with reduced size could not be identified after protoplast isolation.

Therefore, an extensive analysis of cell size in different cells and tissues (freshly prepared or sectioned) was performed. It was found that epidermal cells of the first internode and mesophyll cells of fully expanded leaves in all three transgenic lines were significantly smaller than those in the wt.

It cannot be excluded that the reduction in cell size induced by *enod40* could be the result of an attempt to develop the proper intrinsic organ size as a consequence of increased cell division. Plants are generally believed to

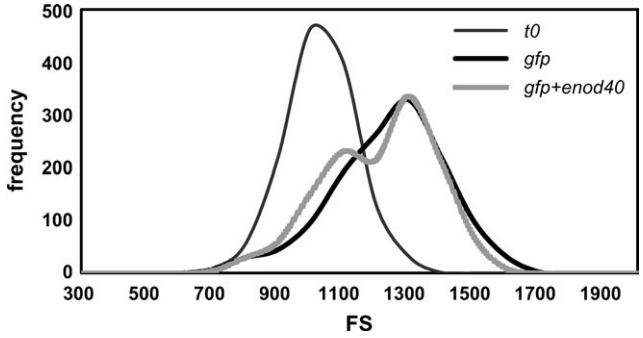


**Fig. 1.** Effect of transient expression of *enod40* on the distribution of FS values in *Arabidopsis* protoplasts. FS values (arbitrary units) were determined at the end of the transformation procedure (t0) and 24 h later in protoplasts transfected with *smrs-gfp* alone and with *smrs-gfp+enod40*. Transformed protoplasts were recognized for their green fluorescence higher than a threshold fixed on the basis of autofluorescence of untransfected protoplasts.

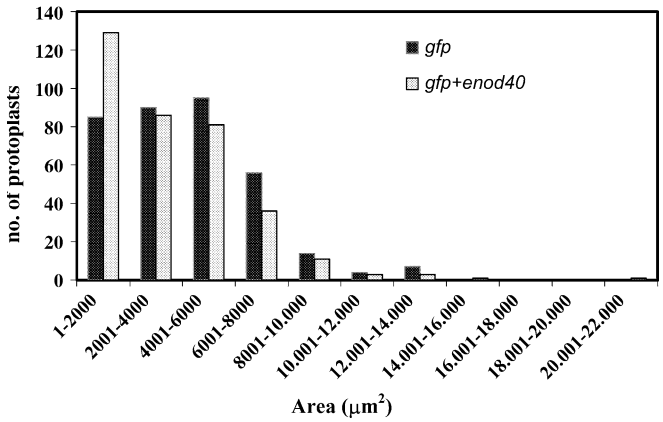


**Fig. 2.** Effect of transient expression of *enod40* on the distribution of protoplast size (area) in *Arabidopsis* protoplasts. The area was determined with Image ProPlus software on 350 protoplasts cultured for 24 h after transfection with *smrs-gfp* alone and with *smrs-gfp+enod40*. One representative experiment is shown.

control organ size by monitoring the size of the entire organ rather than by monitoring the number and size of individual cells (Day and Lawrence, 2000). Therefore a variation in cell number could result in normal-sized organs with altered cell volume. Many authors reported an increase in cell volume compensating a decrease in cell number (Hemerly *et al.*, 1995; Mizukami and Fischer, 2000; Wang *et al.*, 2000). On the other hand, compensation of an increased division rate or division extent with a corresponding decrease in cell volume has never been reported.

A discrimination between the effect on cell division or cell expansion is difficult to achieve in whole plants. Nonetheless, freshly prepared protoplasts cultured in suspension expand before cell division starts again, and could represent a good system with which to determine an independent effect on cell expansion.

Actually, as shown both from FS values and image analysis (Figs 1,2), 24 h after transfection *enod40*-expressing protoplasts were smaller than the control ones. Therefore, the expression of e*nod40* inhibited cell expansion, since within 24 h no cell division had occurred in transfected protoplasts.

**Table 3.** *Mean FS values (arbitrary units) of protoplasts at the end of the isolation procedure (t0) and after 17 h of treatment with* Nt*ENOD40 peptide and unrelated peptides ($10^{-6}$ M)*

Data are from one representative experiment. At least 4000 protoplasts were measured for each treatment.

| | Forward scattering (mean±SE) | Significance of difference with 17 h control |
|---|---|---|
| t0 | 491.3±3.71 | |
| 17 h control | 578.6±2.12 | |
| 17 h *Nt*ENOD40 peptide | 564.2±2.16 | $P< 0.001$ |
| 17 h M1.2 peptide | 575.8±1.66 | Not significant |
| 17 h gramicidin S | 574.4±2.02 | Not significant |

The protoplast system also allowed the effect of exogenous administration of the ENOD40 peptide, which cannot be tested *in planta*, to be studied. Fresh protoplasts treated with tobacco and soybean ENOD40 peptide attained lower FS values with respect to both the untreated control and protoplasts treated with two unrelated peptides.

Furthermore, the latter experiments allowed the conclusion that the effect of *enod40* on cell size can be ascribed, at least in part, to the activity of the peptide, although RNA activity cannot be ruled out. Recently, Campalans *et al.* (2004) showed that *enod40* RNA mediates cytoplasmic relocalization of a nuclear protein in *Medicago truncatula.*

As *enod40* was effective both when the peptide was administered exogenously and when the gene was overexpressed in the cytoplasm, it can be surmised that either receptors or *enod40*-responsive machinery exist both on the plasmalemma and inside the cell or, alternatively, that the peptide is active on the cell surface and its RNA acts in the cytoplasm.

Recently, Hardin *et al.* (2003) found that the ENOD40 peptide antagonizes the *in vitro* phosphorylation of serine 170 of maize sucrose synthase SUS1, which promotes SUS1 proteolysis. *enod40* could therefore preserve the level of SUS1, whose products are utilized in the synthesis of structural polymers, such as cell wall polysaccharides, which are in turn involved in cell expansion. Sugar sensing and signalling are involved in the control of plant growth and development (Rolland *et al.*, 2002). Sucrose itself is considered to be a signal molecule for plants, controlling the expression of genes such as *patatin*, *phloem-specific rol-C*, and the leucine zipper gene *ATB2* (Smeekens and Rook, 1997). The mechanisms behind sucrose-sensing are still largely unknown, although there is evidence that plant cells, rather than monitoring the sucrose concentration directly, may sense changes in the ratio between sucrose and glucose, which is affected by sucrose synthase activity (Sturm and Tang, 1999; Smeekens and Rook, 1997).

Hanson *et al.* (2001) found that the over-expression of the transcription factor HDZhdip in *Arabidopsis thaliana* resulted in sugar-dependent alteration of the development of leaves and cotyledons, which presented larger cells, showing a possible direct effect of a sugar-signalling pathway on cell size control.

All this evidence suggests that *enod40*, by stabilizing sucrose synthase, could trigger a sugar-signalling pathway that modulates cell expansion. *enod40* might also affect cell expansion by directly modulating the activity of other genes or enzymes. It would be interesting to verify whether the expression of expansin genes is affected in transformed cells or in protoplasts treated with the peptide. It is also possible that the development of turgor pressure could be affected.

An additional effect of *enod40* on cell division cannot be ruled out by the present data. An increase in cell division, with normal-sized organs having a higher number of smaller cells, could explain why no clear differences in organ size between all the three transgenic lines and the wt were found despite the differences in cell size.

The effect of *enod40* on cell size was found only in selected types of cells. This finding is probably due to tissues responding differently. Moreover, with regard to protoplasts, only a fraction of transfected protoplasts attained a reduced size. Since only protoplasts expressing the reporter gene were taken into consideration for size and FS analysis, and as these protoplasts should also express *enod40*, it can be surmised that different populations of protoplasts with differing responsiveness to *enod40* expression were present. Studies of the binding of fluorescent ENOD40 peptide to protoplasts could clarify this point.

Along these lines, there is recent evidence that protoplasts isolated from cell suspension cultures are actually a mixture of different populations. In protoplasts from carrot suspension cultures, by cell sorting, it was possible to separate two subpopulations that differ in their cytoplasm: vacuole ratio and morphogenetic potential, which have been interpreted as being derived from 'meristematic' and 'differentiated' cells (Guzzo *et al.*, 2002). Preliminary experiments in *Arabidopsis* suspension cultures also showed that two subpopulations exist that respond differently to 2,4-D (F Guzzo *et al.*, unpublished results).

As far as is known, the reduction of cell expansion shown in the present paper is the first report of a biological effect of *enod40* at the cellular level for a non-legume plant. The phenotype induced by *enod40* reported in this work is somewhat subtle, but it was clearly demonstrated using different, independent approaches. Using reverse genetics, Compaan *et al.* (2003) were unable to show any phenotype in *enod40*-mutated lines. The ability to detect subtle phenotypes depends strongly on the techniques used for evaluation. In the present case, flow cytometric experiments, which allowed the measurement of thousands of cells for each sample, gave highly reproducible and reliable results allowing for the detection of even small differences.

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## References

**Abel S, Theologis A.** 1998. Transient gene expression in protoplasts of *Arabidopsis thaliana.* In: Martinez-Zapater JM, Salinas J, eds. *Methods in molecular biology*, Vol. 82. *Arabidopsis protocols*. Totowa, NJ: Humana Press, 209–217.

**Bechtold NJ, Ellis J, Pelletier G.** 1993. *In planta Agrobacterium*-mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. *Comptes Rendus de l'Academie des Sciences*, *Ser. III, Sciences de la Vie* **316,** 1194–1199.

**Campalans A, Kondorosi A, Crespi M.** 2004. Enod40, a short open reading frame-containing mRNA, induces cytoplasmic localization of a nuclear RNA binding protein in *Medicago truncatula. The Plant Cell* **16,** 1047–1059.

**Charon C, Johansson C, Kondorosi E, Kondorosi A, Crespi M.** 1997. *enod40* induces dedifferentiation and division of root cortical cells in legumes. *Proceedings of the National Academy of Sciences, USA* **94,** 8901–8906.

**Charon C, Sousa C, Crespi M, Kondorosi A.** 1999. Alteration of *enod40* expression modifies *Medicago truncatula* root nodule development induced by *Sinorhizobium meliloti*. *The Plant Cell* **11,** 1953–1965.

**Compaan B, Wey-Cai Y, Bisseling T, Franssen, H.** 2001. ENOD40 expression in the pericycle precedes cortical division in *Rhizobium*–legume interaction and the highly conserved internal region of the gene does not encode a peptide. *Plant and Soil* **230,** 1–8.

**Corich V, Goormachtig S, Lievens S, Van Montagu M, Holsters M.** 1998. Patterns of *ENOD40* gene expression in stem-borne nodules of *Sesbania rostrata*. *Plant Molecular Biology* **37,** 67–76.

**Compaan B, Ruttink T, Albrecht C, Meeley R, Bisseling T, Franssen H.** 2003. Identification and characterization of a *Zea mays* line carrying a transposon-tagged *ENOD40*. *Biochimica et Biophysica Acta* **1629,** 84–91.

**Crespi MD, Jurkevitch E, Poiret M, d'Aubenton-Carafa Y, Petrovics G, Kondorosi E, Kondorosi A.** 1994. *enod40*, a gene expressed during nodule organogenesis, codes for a non-translatable RNA involved in plant growth. *EMBO Journal* **13,** 5099–5112.

**Davis SJ, Vierstra D.** 1998. Soluble, highly fluorescent variants of green fluorescent protein (GFP) for use in higher plants. *Plant Molecular Biology* **36,** 521–528.

**Day SJ, Lawrence PA.** 2000. Measuring dimensions: the regulation of size and shape. *Development* **127,** 2977–2987.

**Flemetakis E, Kavroulakis N, Quaedvlieg NEM, Spaink HP, Dimou M, Roussis A, Katinakis P.** 2000. *Lotus japonicus* contains two distinct ENOD40 genes that are expressed in symbiotic, non-symbiotic, and embryonic tissues. *Molecular Plant–Microbe Interactions* **13,** 987–994.

**Gamborg OL, Miller RA, Ojima K.** 1968. Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research* **50,** 151–158.

**Guzzo F, Cantamessa K, Portaluppi P, Levi M.** 2002. Flow cytometry and sorting of protoplasts from carrot cell cultures reveal two cell subpopulations with different morphogenic potential. *Plant Cell Reports* **21,** 214–219.

**Hardin SC, Tang G-Q, Scholz A, Holtgraewe D, Winter H, Huber SC.** 2003. Phosphorylation of sucrose synthase at serine

170: occurrence and possible role as signal for proteolysis. *The Plant Journal* **35,** 588–603.

**Hanson J, Johannesson H, Engstrom P.** 2001. Sugar dependent alteration in cotyledons and leaf development in transgenic plants expressing the HD Zip gene ATHB13. *Plant Molecular Biology* **45,** 247–262.

**Hemerly AE, Engler A, Bergonioux C, Van Montagu M, Engler G, Inzè D, Ferreira P.** 1995. Dominant negative mutants of the Cdc2 kinase uncouple cell division from iterative plant development. *EMBO Journal* **14,** 3925–3936.

**Kerker M.** 1983. Elastic and inelastic light scattering in flow cytometry. *Cytometry* **4,** 1–10.

**Knud L.** 2004. Molecular cloning and characterization of a cDNA encoding a ryegrass (*Lolium perenne*) ENOD40 homologue. *Journal of Plant Physiology* **160,** 675–687.

**Kouchi H, Hata S.** 1993. Isolation and characterization of novel cDNAs representing genes expressed at early stages of soybean nodule development. *Molecular and General Genetics* **238,** 106–119.

**Kouchi H, Takane K, So RB, Ladha JK, Reddy PM.** 1999. Rice ENOD40: isolation and expression analysis in rice and transgenic soybean root nodules. *The Plant Journal* **18,** 121–129.

**Mizukami Y, Fischer RL.** 2000. Plant organ size control: AINTEGUMENTA regulates growth and cell numbers during organogenesis. *Proceedings of the National Academy of Sciences, USA* **97**, 942–947.

**Murashige T, Skoog F.** 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15,** 473–497.

**Papadopoulou K, Roussis A, Katinakis P.** 1996. *Phaseolus* ENOD40 is involved in symbiotic and non-symbiotic organogenetic processes: expression during nodule and lateral root development. *Plant Molecular Biology* **30,** 403–417.

**Rohrig H, Schmidt J, Miklashevichs E, Schell J, John M.** 2002. Soybean ENOD40 encodes two peptides that bind to sucrose synthase. *Proceedings of the National Academy of Sciences, USA* **99,** 1915–1920.

**Rolland F, Moore B, Sheen J.** 2002. Sugar sensing and signaling in plants. *The Plant Cell* Supplement S185–S205.

**Smeekens S, Rook F.** 1997. Sugar sensing and sugar-mediated signal transduction in plants. *Plant Physiology* **115,** 7–13.

**Sousa C, Johansson C, Charon C, Manyani H, Sautter C, Kondorosi A, Crespi M.** 2001. Translational and structural requirements of the early nodulin gene *enod40*, a short-open reading frame-containing RNA, for elicitation of a cell-specific growth response in the alfalfa root cortex. *Molecular and Cellular Biology* **21,** 354–366.

**Sturm A, Tang GQ.** 1999. The sucrose-cleaving enzymes of plants are crucial for development, growth and carbon partitioning. *Trends in Plant Science* **4,** 401–407.

**van de Sande K, Pawlowski K, Czaja I, *et al*.** 1996. Modification of phytohormone response by a peptide encoded by ENOD40 of legumes and a nonlegume. *Science* **273,** 370–373.

**Vleghels I, Hontelez J, Ribeiro A, Fransz P, Bisseling T, Franssen H.** 2003. Expression of *enod40* during tomato plant development. *Planta* **218,** 42–49.

**Wang H, Zhou Y, Gilmer S, Whithwill S, Fowke LC.** 2000. Expression of the plant cyclin-dependent kinase inhibitor ICK1 affects cell division, plant growth and morphology. *The Plant Journal* **24,** 613–623.

**Yang WC, Katinakis P, Hendriks P, Smolders A, de Vries F, Spee J, van Kammen A, Franssen H.** 1993. Characterization of *GmENOD40*, a gene showing novel patterns of cell-specific expression during soybean nodule development. *The Plant Journal* **3,** 573–585.