Journal of Plant Physiology 171 (2014) 329–339

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Physiology

Variable expansin expression in *Arabidopsis* leads to different growth responses

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ARTICLE INFO

Article history:
Received 16 August 2013
Received in revised form
18 September 2013
Accepted 18 September 2013
Available online 19 October 2013

Keywords:
Arabidopsis
Expansin
Hypocotyl
Leaf development
Petiole

ABSTRACT

Expansins have long been implicated in the control of cell wall extensibility. However, despite ample evidence supporting a role for these proteins in the endogenous mechanism of plant growth, there are also examples in the literature where the outcome of altered expansin gene expression is difficult to reconcile with a simplistic causal linkage to growth promotion. To investigate this problem, we report on the analysis of transgenic *Arabidopsis* plants in which a heterologous cucumber expansin can be inducibly overexpressed. Our results indicate that the effects of expansin expression on growth depend on the degree of induction of expansin expression and the developmental pattern of organ growth. They support the role of expansin in directional cell expansion. They are also consistent with the idea that excess expansin might itself impede normal activities of cell wall modifications, culminating in both growth promotion and repression depending on the degree of expression.

Introduction

Expansins are plant cell wall proteins which have long been implicated in the control of cell wall extensibility and, thus, in the control of plant growth (Cosgrove, 2005). Leading on from the initial purification and characterization of expansins in cucumber hypocotyl (McQueen-Mason et al., 1992), many authors have shown that expansins are abundant in rapidly growing tissue/organs. These include rice internodes (Cho and Kende, 1997; Lee and Kende, 2001), rice root hairs (Yu et al., 2011), oat coleoptiles (Cosgrove and Li Zhen, 1993), cotton fibers (Orford and Timmis, 1998), and elongating roots in soybean (Lee et al., 2003) and maize (Kam et al., 2005). Expansins are also involved in organogenesis, including leaf initiation in tomato (Reinhardt et al., 1998), and root hair initiation in pine (Hutchison et al., 1999) and *Arabidopsis* (Cho and Cosgrove, 2002). Furthermore, expansins play a role in non-growing tissues, often involving elements of cell wall differentiation. This includes ripening in various fruits (Brummell et al., 1999b; Harrison et al., 2001; Hiwasa et al., 2003; Sane et al., 2005; Yoo et al., 2003), differentiation of xylem cells in zinnia (Milioni et al., 2001) and hybrid aspen (Gray-Mitsumune et al., 2004), and in poplar wood-forming tissue (Sterky et al., 1998).

In addition to correlative data, a number of papers have reported on the manipulation of expansin gene expression using transgenic methods in rice (Choi et al., 2003), tobacco (Pien et al., 2001; Sloan et al., 2009; Wang et al., 2011), tomato (Brummell et al., 1999a; Caderas et al., 2000), petunia (Zenoni et al., 2004, 2011) and *Arabidopsis* (Cho and Cosgrove, 2000; Goh et al., 2012). For example, the overexpression of endogenous expansin genes has been associated with increased coleoptile and mesocotyl growth in rice (Choi et al., 2003), and extended leaf petioles in *Arabidopsis* (Cho and Cosgrove, 2000). Although some of these reports have provided data essentially supporting a role for expansin in growth, it is noticeable that at least some reports suggest that a simple linkage between expansin gene expression and growth is not valid. For instance, the correlation found in specific up-regulated expansin gene expression association with leaf expansion in maize (Muller et al., 2007) was not found in the case of fescue leaves (Reidy et al., 2001) or tomato hypocotyls (Caderas et al., 2000). Indeed, the overexpression of an expansin isoform in tomato led to a repression in hypocotyl growth (Rochange et al., 2001). Thus, the precise relationship of expansins and plant growth remains unclear or at least variable.

Previously we reported on a series of experiments in which inducible overexpression of a cucumber alpha-expansin (EXPA) gene in tobacco led to an increased growth of the leaves, but only during a specific window of development (Sloan et al., 2009). These

Abbreviations: DAS, day after sowing; Dex, dexamethasone; DMSO, dimethyl sulfoxide; EXPA, alpha-expansin; GUS, β-glucuronidase.

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http://dx.doi.org/10.1016/j.jplph.2013.09.009

data supported the hypothesis that final leaf size is correlated with the maximal rate of leaf expansion (Cookson et al., 2005), which occurs during the phase of maximal growth sensitivity to induced expansin expression. This has also been substantiated by recently published results showing that the effects of suppressed multiple endogenous expansin gene expression depended on leaf developmental stage, with different responses between the leaf blade and petiole (Goh et al., 2012). We postulated that this differential sensitivity to expansin activity during development might account for some of the disparate data in the literature.

To further investigate this problem, we have generated a series of transgenic *Arabidopsis* plants in which the well-characterized heterologous cucumber EXPA gene can be inducibly overexpressed in *Arabidopsis*. Our data indicate that although (as in tobacco) induced expansin expression can lead to an increase in lamina area, there was a more dramatic effect of decreased petiole growth following high-level induction of expansin expression. This suppression of extension growth was also observed in the hypocotyls of these plants. These data indicate that the outcome of altered expansin gene expression is context-dependent, both in terms of organ and perhaps growth habit. They are consistent with the hypothesis that expansin acts to unlock elements within the cell wall to allow access and action of other cell wall components and that it is the nature of these co-acting molecules that determines the final phenotype observed.

Materials and methods

Plant growth conditions and treatments

Arabidopsis thaliana (L.) Heynh. (ecotype Col-0 from Nottingham Arabidopsis Stock Centre) seeds were surface-sterilized and stratified in the dark at 4 °C for 1 week before being sown aseptically on solid media of 0.5× MS salts (Murashige and Skoog, 1962) (Sigma–Aldrich), 1% (w/v) sucrose, and 0.8% (w/v) plant agar (Duchefa Biochemie) in 12 cm × 12 cm square petri dishes (Greiner Bio One) with 36 seeds/plate. Growth conditions were 22 °C day/20 °C night under a 16-h photoperiod, light intensity of 100 μmol m^{-2} s^{-1}.

For the induction of pOpON::CsEXPA1 transformant plants, growth media were supplemented with various concentrations of dexamethasone (Dex) prepared from 10 mM stock in dimethyl sulfoxide (DMSO) diluted using distilled water. Control media were supplemented with an equivalent concentration of DMSO (0.1% (v/v)). For staged-transfer experiment of gene expression study, plants grown on solid media at a density of 4 cm^2 per seed were categorized according to the number of the number of visible leaves (>1 mm width) and the size of leaf 5. Plants of equivalent developmental stage at 14 days after sowing (DAS) were chosen to be transferred to new small round petri dishes (5 cm diameter, $n = 3$ each) containing solid media supplemented with various concentrations of Dex for 24 h induction. For time-course study of dark-grown hypocotyl and root growth, seeds were grown at a density of 70 seeds per plate over three rows on 0.5× MS agar medium supplemented with 10 μM Dex or DMSO 0.1%. The petri dishes were double wrapped in aluminum foil.

Histochemical β-glucuronidase (GUS) assays of induced plants/leaves/hypocotyls were performed according to the standard protocols (Jefferson et al., 1986).

Generation of pOpON::CsEXPA1 transformant plants

The coding sequence *CsEXPA1* (992 bp) from cucumber (Cucsa.143960.1) was cloned into a Dex-inducible gene expression system under the control of bidirectional pOp6 promoter through TOPO cloning to generate transgenic *Arabidopsis* (pOpON::CsEXPA1) of Columbia-0 (Col-0) background. TOPO cloning was performed according to the manufacturer's protocol (Invitrogen) and the product was used for heat transformation of DH5α chemically-competent *Escherichia coli* (Bioline). Inserts from pENTR/D-TOPO were recombined using LR clonase II (Invitrogen) into a Gateway-compatible binary vector pOpON2.1 (Wielopolska et al., 2005). Vector was transformed into *Agrobacterium tumefaciens* strain GV3101::pMP90RK through electroporation and then wild-type Col-0 *Arabidopsis* plants grown in Levingston M3 compost by the floral dip method (Clough and Bent, 1998). T1 progeny were self-pollinated, and subsequently selected using the appropriate antibiotic (50 μg/mL kanamycin), and genotyped using specific primers. Induction tests and histochemical GUS assays were performed in subsequent generations to select for T3 homozygous transformants with stable expression pattern upon induction. Single T3 homozygous transformant line 10/1 was used in this study.

Molecular characterization of pOpON::CsEXPA1 transformant plants

RNA extracted from whole-plant tissue using TRIZOL method (Chomczynski and Sacchi, 1987) were standardized to equal amount (4 μg) through spectrophotometric measurement and gel electrophoresis and used as template for the first-strand cDNA synthesis using M-MLV reverse transcriptase Rnase H minus (Promega) with oligo dT(18). Products were used as template for subsequent PCR amplifications. Semi-quantitative RT-PCR analysis of pOpON::CsEXPA1 used forward primer 5′-TTTGTCTTCACCTTCGCTGA-3′ and reverse primer 5′-GCCTTGGCCATTGAGATAGT-3′ for *CsEXPA1*, with BIOTAQ DNA polymerase (Bioline): initial denaturation at 94 °C for 5 min, cycles: 15 s at 94 °C, 30 s at 60 °C, 1 min at 72 °C, and 1 min final extension at 72 °C. The PCR cycle during the exponential phase was paused at defined intervals at the end of extension and aliquots were routinely taken at cycle 24, 28, 30 and 32. All PCR reactions were performed with *RBCS* (At5g38410) forward primer 5′-CTTCCTCTATGCTCTCCTCC-3′ and reverse primer 5′-GTTGTCGAATCCGATGATCC-3′, and *PP2A* (At1g13320) forward primer 5′-ACGTGGCCAAAATGATGCAA-3′ and reverse primer 5′-CGCCCAACGAACAAATCACA-3′ as internal controls. RT-qPCR experiments using SYBR® Green PCR master mix (Applied Biosystems) in 96-well optical reaction plates with optical adhesive covers (ABI PRISM™) were designed, performed and analyzed using a StepOnePlus™ Real-Time PCR system with its accompanying StepOne Software (Applied Biosystems, version 2.2). Primers were designed using QuantPrime (http://www.quantprime.de/). The PCR efficiencies of each primer pair (200–250 nM working concentrations) were determined from standard curve experiments using five 10-fold serial dilutions of cDNA to a final reaction volume of 20 μL. *CsEXPA1* forward primer 5′-CCTCCTCTCCAACATTTCGAC-3′ and reverse primer 5′-TGGTACCCTACGAAAGGAGAC-3′, and *UBC21* (At5g25760) forward primer 5′-CTCTTAACTGCGACTCAGGGAATC-3′ and reverse primer 5′-TGTGCCATTGAATTGAACCCTCTC-3′ were used in comparative C_T experiments for quantitative analysis of *CsEXPA1* gene expression level. All results were determined from three biological replicates, each with three technical replicates.

Protein analysis of pOpON::CsEXPA1 transformant plants

Cell wall protein extraction was performed as described previously (Rochange et al., 2001) using growing whole plant (21 DAS) from 10 μM Dex-treated and 0.1% (v/v) DMSO mock-treated wild-type and transgenic *Arabidopsis* plants. 10 μg of cell wall protein extracted from pooled samples of 36 plants (21 DAS) was loaded per well for immunodetection on protein blots. Proteins were separated in 12% SDS-PAGE gels then blotted onto