Plant Science 239 (2015) 216–229

Contents lists available at ScienceDirect

Plant Science

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

# *Arabidopsis BNT1*, an atypical *TIR–NBS–LRR* gene, acting as a regulator of the hormonal response to stress

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ARTICLE INFO

*Article history:*
Received 2 May 2015
Received in revised form 20 July 2015
Accepted 25 July 2015
Available online 30 July 2015

*Keywords:*
Arabidopsis
Abiotic stress
Biotic stress
BURNOUT
TIR–NBS–LRR
Plant hormone

ABSTRACT

During their life cycle, plants have to cope with fluctuating environmental conditions. The perception of the stressful environmental conditions induces a specific stress hormone signature specifying a proper response with an efficient fitness. By reverse genetics, we isolated and characterized a novel mutation in Arabidopsis, associated with environmental stress responses, that affects the *At5g11250/BURNOUT1 (BNT1)* gene which encode a Toll/Interleukin1 receptor–nucleotide binding site leucine-rich repeat (TIR–NBS–LRR) protein. The knock-out *bnt1* mutants displayed, in the absence of stress conditions, a multitude of growth and development defects, suchas severe dwarfism, early senescence and flower sterility, similar to those observed *in vitro* in wild type plants upon different biotic and/or abiotic stresses. The disruption of *BNT1* causes also a drastic increase of the jasmonic, salicylic and abscisic acids as well as ethylene levels. Which was consistent with the expression pattern observed in *bnt1* showing an over representation of genes involved in the hormonal response to stress? Therefore, a defect in *BNT1* forced the plant to engage in an exhausting general stress response, which produced frail, weakened and poorly adapted plants expressing "burnout" syndromes. Furthermore, by *in vitro* phenocopying experiments, physiological, chemical and molecular analyses, we propose that *BNT1* could represent a molecular link between stress perception and specific hormonal signature.



## 1. Introduction

Plants are constantly confronted by diverse environmental stresses, which modify their metabolism, growth and productivity. The capacity to respond to these stresses is critical for plant survival, proper development and robustness of plant traits. Therefore, plants have evolved multiple defence, escape mechanisms [1,2] as well as the use of plant stress hormones, such as, jasmonic (JA), salicylic (SA) and abscisic (ABA) acids and ethylene (ET), which mediate host responses to many environmental stresses [3–5]. Differential expression of stress-related genes following various abiotic and biotic stresses or plant hormone treatments suggests there is a crosstalk between the signalling transduction pathways elicited by biotic stresses and plant hormones [4,6–8]. Indeed, current thinking is that SA, JA, ET and ABA pathways do not function independently, but influence each other through a complex network of regulatory interactions and crosstalk to fine-tune plant defence, suggesting a great complexity of interactions [4,9]. Moreover, it is increasingly clear that plants use hormonal signals in a synergistic manner to achieve distinct responses to an environmental stress, and that multiple hormone response pathways interact to translate initial perception events into the appropriate response that improves plant fitness in the presence of diverse pathogens [10]. Therefore, for each stress, a specific hormonal signature is elaborated by the plant to address a specific defence response, ensuring plant survival [11]. A better understanding of this process would give insight not

*Abbreviations:* BNT1, BURNOUT1; TIR–NBS–LRR, toll/interleukin1 receptor-nucleotide binding site leucine-rich repeat; JA, jasmonic acid; SA, salicylic acid; ABA, abscisic acid; ET, ethylene; WT, wild type; T-DNA, transferred DNA.

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http://dx.doi.org/10.1016/j.plantsci.2015.07.017
0168-9452/

only into possible ecological and evolutionary responses in wild species, but this would be useful also for engineering crop species.

In order to go further on the molecular aspects of the plant stress response, we use, from our *Arabidopsis* T-DNA insertion library [12,13], a recessive mutant with altered plant stress hormone levels, which was designated as *burnout1* (*bnt1*). The mutant, in addition to its high stress hormones levels, displayed a multitude of growth and developmental defects such as severe dwarfism, round-shaped leaves, early senescence and flower sterility (the "*burnout*" syndrome). Using positional cloning, the *BNT1* gene was identified as a *TIR–NBS–LRR* gene, which belongs to the *R* gene family. We found that the *bnt1-1* phenotype is very close to those observed in wild type (WT) plants under environmental variations, and therefore, *bnt1-1* constantly expressed a stressed phenotype. According, *BNT1* represent a fine tuner of the hormonal stress signature and our findings bring a robust and fresh molecular perspective to the genetic control of stress response.

## 2. Materials and methods

### 2.1. Plant material

*Arabidopsis* plants were grown as described previously [14]. For quantifying root growth, *Arabidopsis* seeds (C24 and Col-0) were plated on 0.5 MS medium with 1% sucrose and 0.8% agar (pH5.9) and stratified for 2 days at 4 °C. Seedlings were grown on vertically oriented plates in growth chambers under a 16-hr-light/8-hr-dark photoperiod at 22 °C. For dark-grown seedlings, the plated seeds were kept in the dark. The hypocotyl lengths of 4- and 14-day-old WT and mutant plants were measured for etiolated and light grown. The mutant plants were numbered and sub -cultured on 0.5 MS medium for 4 to 6 weeks under standard conditions to ensure that they corresponded to *bnt1-1* mutant. In greenhouse, plants were incubated at 22 °C with a 16-hr-light/8-hr-dark photoperiod. For soil dehydration assays, plants were grown for 21 days, and were then grown under the same conditions with or without additional water for 21 days. For each experiment and analysis the results are presented as means with standard error bars and all claimed comparisons are based on statistical evaluations with *t* test ($^{*}p < 0.05, ^{**}p < 0.01, ^{***}p < 0.001$). Experiments were repeated at least 3 times.

### 2.2. Pharmacological and hormonal treatments

1-naphthaleneacetic acid (NAA), *N*-(1-naphtyl)phtalamic acid (NPA), 1-aminocyclopropane-1-carboxylate (ACC), aminoethoxyvinylglycine (AVG), *N*6-benzyladenine (BA), paclobutrazol (Paclo), diethyldithocarbamic acid (DETC), methyl jasmonate (MeJA), salicylic acid (SA); abscisic acid (ABA), cobalt chloride ($CoCl_2$), silver nitrate ($AgNO_3$), bacterial flagellin-derived flg22 peptide (Flg22), fumonisin FB1 (FB1) were filter sterilized and added to the autoclaved medium, as indicated in the Figures and Tables.

### 2.3. Phenotypical analysis, microscopy and statistics

Plant phenotypes were imaged with a binocular microscope (Leica) associated with digital camera. For the root length analysis, plates were scanned on a flat-bed scanner (Epson). For scanning electron microscopy (SEM), tissues were fixed in 1% glutaraldehyde (0.1 M) cacodylate buffer, post-fixed in 1% osmium tetraoxide and dehydrated in a graded ethanol series. Dehydrated material was dried to a critical point in liquid carbon dioxide, and gold-coated specimens were mounted on a scanning electron microscope (Philips 505) at 20 kV acceleration [12]. For the confocal laser scanning microscopy, a FV10 ASW confocal microscope (Olympus) was used. Seedlings were stained for 5 min in 0.01 mg ml $^{-1}$ propidium iodide. Plant traits parameters (roots, hypocotyl lengths, stomata number and density) were analysed with the ImageJ software (National Institutes of Health). Images were processed in Adobe Photoshop CS6 (Adobe Systems).

### 2.4. Determination of ET, ABA, JA and SA production

The quantitative determination of ET, using gas chromatography, has been described previously [13]. ET was assayed by a gas chromatography (Carlo Erba 4100) equipped with a propack N column (800 × 5 mm; Dionex) and a flame ionization detector. Statistical analyses to determine significant differences were assayed using the Mann–Whitney *U*-test [15]. ABA, JA and SA quantifications were performed as described [16]. C24, *bnt1-1* and SALK lines, stressed or not, were harvested into liquid nitrogen and freeze dried. Samples were grounded in a bead beater (Retsch MM400) with 3 mm tungsten beads at 25 Hz/s for 3 min. Ten milligrams of powdered tissue were weighed and extracted with 10% methanol containing 1% acetic acid. Each treatment also included an extraction control containing no plant material. Samples were then analysed by HPLC-electrospray ionisation/MS–MS using an Agilent 1100HPLC coupled to a Q-Tof Ultima Global (Proteome Works System). Chromatographic separation was carried out on a Shimadzu HPLC column XR-ODS 2.2 μm 100 × 2 mm column. For each measurement, three independent experiments were performed. Statistical analyses were performed using Student's *t*-test ($^{**}p < 0.01$).

### 2.5. Chlorophyll and anthocyanin measurements

Fresh leaves (500 mg) were ground in cold 80% acetone, kept 30 min at 0 °C in the dark and centrifuged. Chlorophyll content (μg/ml) was determined by measuring the absorbance at 663 nm and 645 nm [17]. To detect anthocyanin level, the fresh leaves were ground in acidified methanol (0.1% v/v) and agitated 10 min at 4 °C in darkness. The methanolic extract filtrated, which contained anthocyanins, was analysed by measuring the absorbance at 530 nm and 657 nm [18].

### 2.6. Southern analysis and mapping mutation

DNA extraction and positional cloning of the *BNT1* locus was done as described previously [14]. Primers used for *bnt1-1* mapping are listed in Table S5. Southern blot analysis was performed on 10 μg DNA digested by *Bcl*I, *Nde*I, *Sph*I and *Sac*I+*Nde*I, blotted onto Hybond N$^{+}$ membranes according to the manufacturer (Amersham) and hybridized to two different probes derived from the GUS region (1.8 kb *Eco*RV-*Xba*I fragment; specific to the T-DNA) and the vector region (2.3 kb *Nde*I-*Nde*I fragment; specific to the origin of replication PVS1) from the pGS Gluc1 plasmid. Probes were generated by the random primer method with [α-P$^{32}$] dCTP using Oligolabelling kit (Pharmacia).

### 2.7. Quantitative RT-PCR

RNA was extracted using the RNeasy kit (Qiagen) from whole seedlings 14 days-old. The RNase-free DNase Set (Qiagen) was carried out for 15 min at 25 °C to perform a DNase treatment. From 1 μg total RNA, the iScript$^{TM}$cDNA Synthesis Kit (Biorad) was used to prepare Poly(dT) cDNA which were quantified using the LightCycler 480 SYBR GREEN I Master mix (Roche) and a LightCycler 480 (Roche) according to the manufacturer's instructions. PCR was carried out in 384-well optical reaction plates heated for 10 min to 95 °C to activate the hot start Taq DNA polymerase, followed by 40 cycles of denaturation for 60s at 95 °C and annealing/extension