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Illumination of Arabidopsis roots induces immediate burst of ROS production

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Arabidopsis roots are routinely exposed to light both during their cultivation within transparent Petri dishes and during their confocal microscopy analysis. Here we report that illumination of roots which naturally grow in darkness, even for a few seconds, induces an immediate and strong burst of reactive oxygen species (ROS). Plant scientists studying roots should pay great attention to the environment of living roots, and keep them in darkness as long as possible. Results obtained using illuminated roots during in vivo microscopic analysis should also be interpreted with great caution.

Introduction

Plant roots in nature grow within soil in more-or-less complete darkness. This conventional wisdom seems to be forgotten in the contemporary plant sciences. In the former studies, attention to root part has not been sufficient, partly because many plant scientists believe that as roots always grow in the full darkness underground, the roots are "ignorant" of any light either during their growth in natural situation, or in the laboratory conditions, as well as in their evolutionary history. Nevertheless, the fact is that plant roots are extremely sensitive to light[1] and that this sensitivity is important for their optimal performance if grown outside in soil.[2] Ever since Arabidopsis has been brought to laboratories and showed that it can be grown in the transparent Petri dishes by George P. Rédei some 50 years ago,[3,4] Arabidopsis roots are routinely exposed to light during preparation for their analysis without comparing data obtained from the dark-grown controls. As a consequence, almost all studies published using *Arabidopsis thaliana* have used roots illuminated during seedling growth. These roots have been obtained using Arabidopsis seedlings grown in transparent Petri dishes placed within plant growth chambers with a 16h/8h light/dark regime. This light exposure of roots is potentially stressful not only for roots but also for the seedlings, as ROS-based stress signals accomplish rapid and systemic transfer throughout the whole plant body.[5,6] Arabidopsis roots respond to diverse stressful situations with increased growth rates,[7] so called "escape tropism" strategies[8-10] affecting data obtained with illuminated roots. For example, in the dark-grown roots, the auxin efflux facilitator PIN proteins[11] are not polarly localized at the plasma membrane but accumulate within endosomes/vacuoles.[7] Blue-light signaling is required for the plasma membrane targeting of these proteins[7] critical for growth, polarity, development, and morphogenesis of plants.[11] Plant cells execute tight control of their redox homeostasis.[12] They possess an array of antioxidant mechanisms to cope with high rates of ROS production. Almost any stress results in rapidly enhanced productions of ROS due to disrupted balance of the cellular redox status.[12] In this report, the instant production of ROS after the illumination to Arabidopsis root was documented and this light-induced ROS induces root escape via negative root phototropism.

Results and Discussion

As **Figure 1A** shows, dark-grown shoot starts to grow quicker for seeking for the light source, which is a well known phenomenon of skotomorphogenesis.[13,14] On the other hand, illumination of roots stimulates their growth as roots try to escape from light, resulting in imbalance of the root-shoot ratio which in normal physiological situation is approximately 1:1. This light-induced increase in the root growth, combined with the negative root phototropism, can be regarded as a physiologically relevant response, as it would, outside in the nature, allow the illuminated roots to get back into darkness (soil). Importantly in this respect, the soil grown Arabidopsis seedlings (having their roots within more-or-less complete darkness) show 1:1 root-shoot ratio. This increased root growth rate combined with negative root tropism can be considered for the root escape tropism (negative phototropism) (**Fig. 1B**). This suggests that roots have powerful mechanism to increase their growth rate or movement direction in response to ambient light. When the seedlings are cultivated in soil, the proportion of shoot and root is almost 1:1 (**Fig. 1C**), suggesting that the high speed of root growth is not required in their natural environment.

In **Figure 1E**, newly designed split Petri dish system, in which the lower part of petri dish is covered by black tape and aluminum foil is shown. The motivation for designing this system is to accomplish an ideal growing situation in laboratory by keeping

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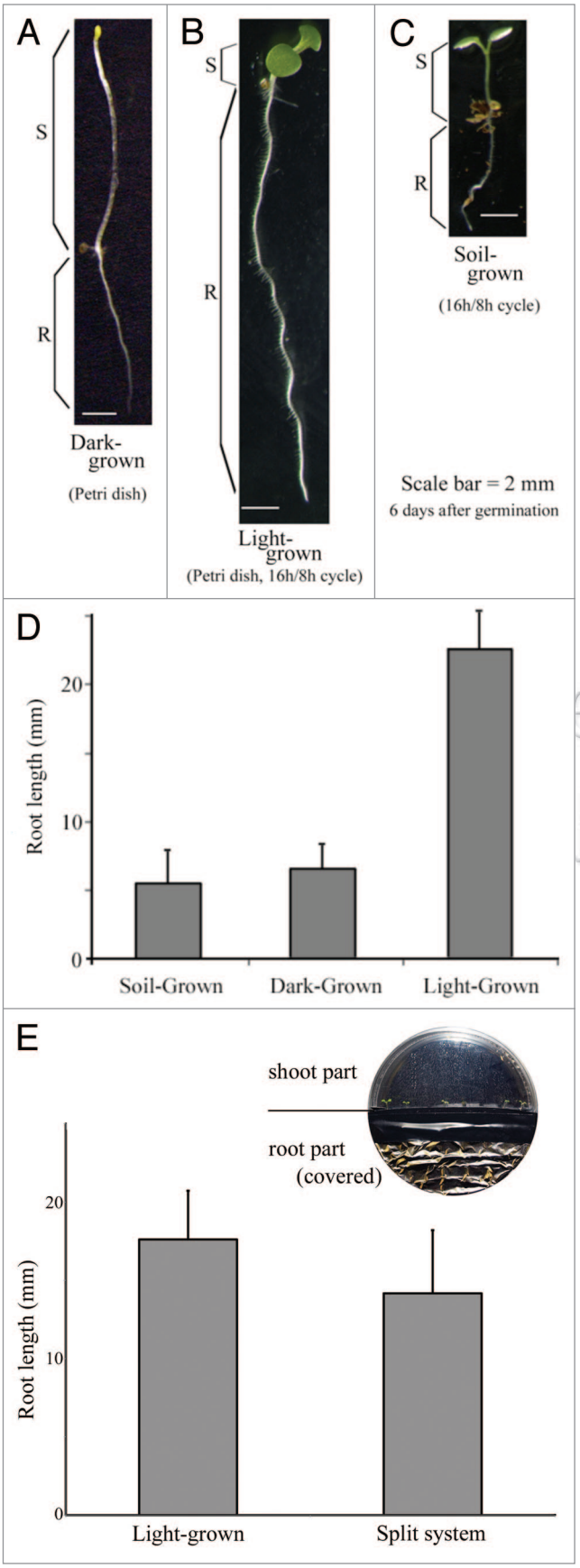

only the root part in darkness. As a result, skotomorphogenesis of shoot and increase of root length has not been observed by this split system. However, this system is still not optimal and slight amount of light (~10 μmol m^{-2} s^{-1}) penetrates into the root part of darkenned Petri dishes and stimulates light responses of roots. It should be improved further. In addition, it was observed that expression level of certain protein, such as annexin AnnAt4, is altered dramatically in soil-grown instead of light-grown Arabidopsis roots grown in transparent Petri dishes.[15] Besides visible light, roots are very sensitive also to UV-B light with root apex being the most sensitive part of plant roots.[16-19] Recent study has reported that UVR8 protein is the UV-B receptor both in roots and shoots.[20]

In **Figure 2**, the generation of hydrogen peroxide stained by DAB in the root apex region after 60 min of both white and blue light illumination. While very low intensity of DAB staining was observed in the root apex without illumination, the results showed that hydrogen peroxide was produced rapidly under root illumination. In addition, precipitation of formazan in root apex region was observed after 15 min blue light illumination by NBT-staining (data not shown).

The data shown in **Figure 3A** documents instant ROS production after 10 sec of blue light illumination detected by the fluorescence probe, Peroxy-Yellow 1 Methyl Ester (PY1-ME), which allows sensitive detection of intracellular hydrogen peroxide.[21] Since PY1-ME shows very high resolution and reactivity,[21] almost instant production of ROS was detected using this probe in roots of Arabidopsis. The intensity of PY-1 fluorescence diminished at 5–10 min after 10 sec of root illumination. After the fluorescent signal has vanished, only few seconds of illumination can provoke the ROS-generation again. In addition, the results of treatment with several ROS-related enzyme-inhibitors and scavengers shown in **Figure 3B** suggested the generation of superoxide after illumination of roots. The exposure of roots to diphenyliodonium (DPI) results effective inhibition of NADPH oxidase which is located on the plasma membrane and produces the superoxide by reducing oxygen. Diethyldithiocarbamate (DCC) inhibits superoxide dismutase (SOD) which converts superoxide to hydrogen peroxide. Since both inhibitors reduced PY1-ME fluorescence, superoxide emerges as a possible ROS species generated after illumination of roots. Moreover, both 4-hydroxy-TEMPO, which is broadly used as the spin trap agent for electron spin resonance (ESR) and tiron, was used for scavenging superoxide[22] and induced the reduction of PY1-EM fluorescence. Salicylic hydroxamic acid (SHAM), inhibitor of peroxidase, showed no effect on ROS production after root illumination, suggesting that peroxidase have no relationship with these rapid root responses to light. 2′, 7′-Dichlorofluorescein

Figure 1. (A) Dark grown (etiolated) seedling. (B) Light-grown seedling. (C) Soil-grown seedling. (D) Comparison of root length. Light and Dark cycle is 16 hours/ 8 hours. Each bar indicates standard error (n = 12). (E) Comparison of root length between light-grown (n = 23) and only root part covered (n = 80) using split Petri dish system. Difference of length was significant by t-test.

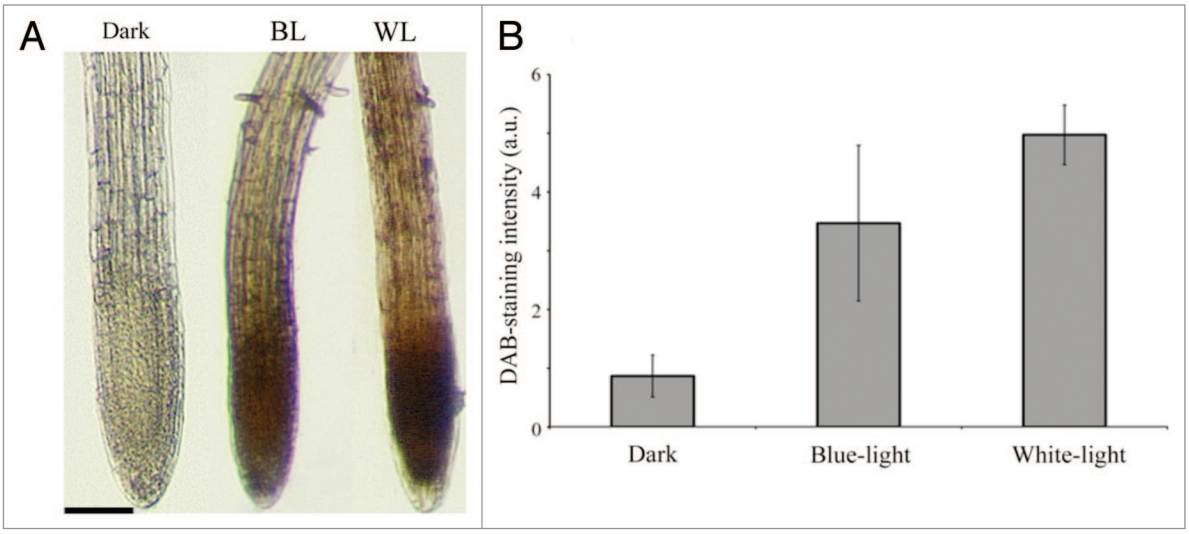

Figure 2. (A) DAB staining with 60 min illumination. BL; Blue light illumination, WL; White light illumination. (B) Comparison of DAB-staining intensity between darkness, blue light and white light illumination. Each bar indicates standard error (n = 6). Scale bar indicates 100 μm.

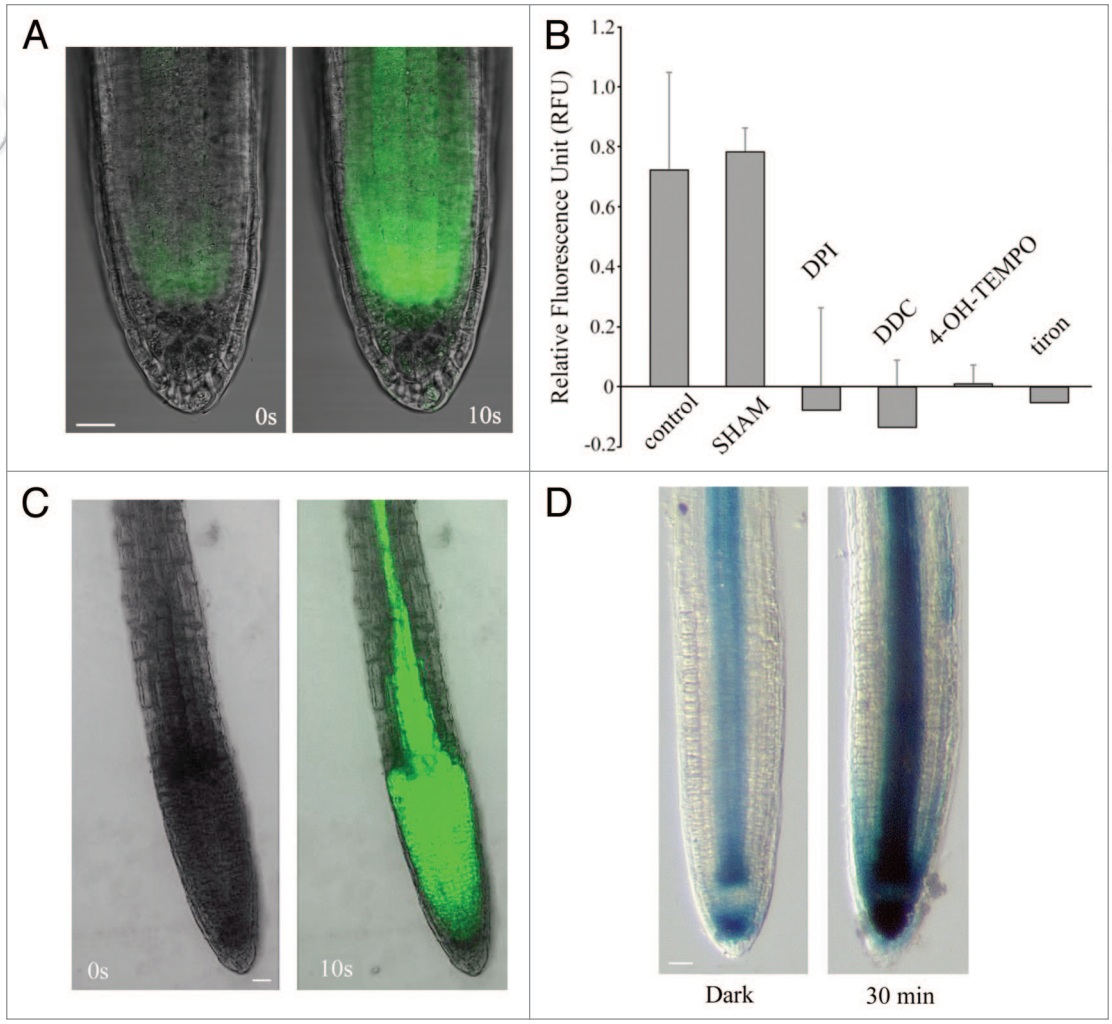

Figure 3. (A) Immediate ROS production after 10 sec Blue light illumination detected by PY-1 (peroxy-yellow 1). (B) Treatment of enzyme inhibitors and ROS scavengers. Each bar indicates standard error (n = 3). Asterisk indicates significant difference. (C) Increase of DCF fluorescence after 10 sec blue light illumination. (D) Up-regulation of DJ-1:GUS expression following 30 min blue light illumination. Scale bars indicate 25 μm.

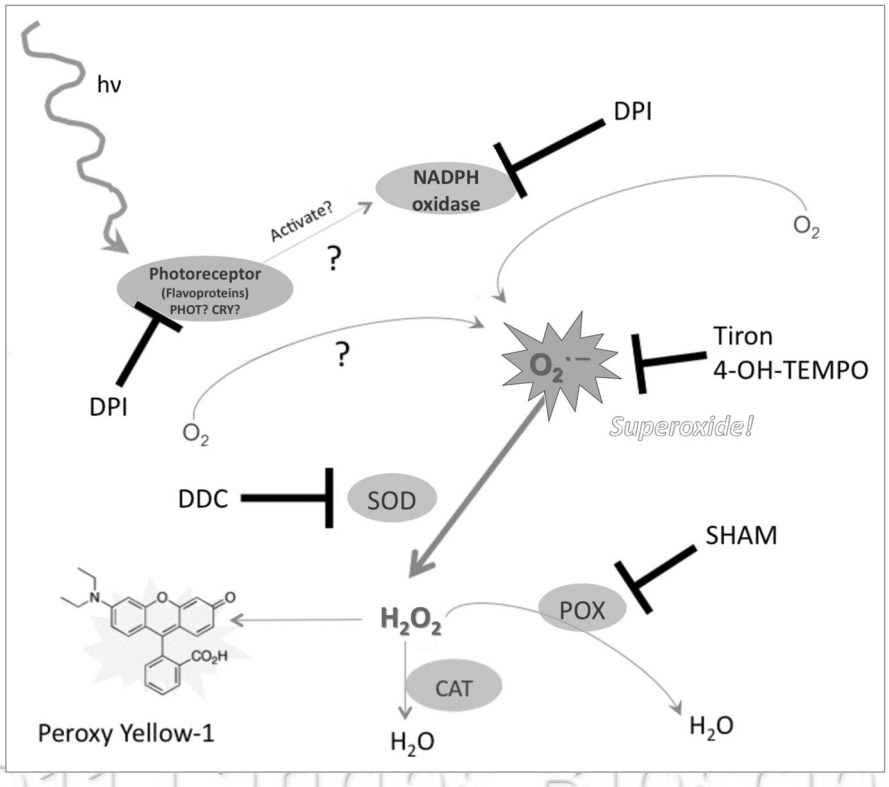

Figure 4. Hypothetical mechanisms of light induced ROS (superoxide)-generation in roots.

diacetate ($DCFH^2$-DA) is conventionally used for detecting intracellular hydrogen peroxide (emission: 488 nm and excitation: 525 nm). We have also observed instant production hydrogen peroxide, already after 10 sec of blue light illumination, using this probe (**Fig. 3C**). In **Figure 3D**, we used GUS line of Arabidopsis DJ-1 (AtDJ-1) which was identified as a homolog of Human DJ-1 PARK7.[23] Mutation of human DJ-1 causes Parkinson's disease resulting in neuronal death. It was previously reported that endogenous AtDJ-1 level in leaf was increased after treatment with stress factors, such as strong light, H^2O^2, methyl viologen and copper sulfate.[23] DJ-1 supports the cellular redox homeostasis by subserving a SOD-activity in both mammalian and plant cells.[23] Here we have found, following the GUS staining protocol of Vitha et al.,[24] increased DJ-1::GUS expression in root apices of Arabidopsis in response to short (30 min) blue light exposures (**Fig. 3D**). Taken together, hypothetical mechanism of the ROS production after the illumination of roots is summarized in **Figure** 4. Since DPI inhibits not only NADPH oxidase but also general flavoproteins including photoreceptors such as phototropins, cryptochromes and so forth; the exact source of superoxide cannot be pointed out here. Therefore, it is still obscure how superoxide is produced in the root apex by light.

Even extremely short—in fact, few seconds (**Figure 3A–C**)—light exposure of roots induces a stress situation for these delicate plant organs as evidenced by the immediate burst of ROS production. It has been suggested that the ROS may play an important role as primary cellular signal molecule in plants. In fact, it might represent the driving force for escape of roots from unfavorable light conditions.[8-10] Recently, it has been reported that ROS balance among superoxide and H_2O_2 in the root apex region control cell proliferation and elongation, respectively.[25] These ROS signals are known to be transported rapidly and systematically from roots to shoots[26] as well as throughout the plant tissues and organs.[5,6,27] In conclusion, plant scientists should pay great attention to the environment of living roots, keeping them in darkness as long as possible. If results are obtained with illuminated roots due to handling procedures and/or in vivo microscopic analysis, they should be interpreted with a great caution, keeping in mind that only a few seconds of illumination induces immediate and abundant ROS production in Arabidopsis root cells (**Fig. 3**). It is essential to perform proper dark-grown control by exposing to the usual 16/8 light/dark cycles only the shoots, keeping the roots in darkness (optimally in growing the Arabidopsis seedlings in pots, having all roots within soil). Data obtained using the illuminated Arabidopsis roots in the last three to four decades should be carefully re-interpreted.

Materials and Methods

Plant materials. Arabidopsis (*Arabidopsis thaliana L.*) seeds were soaked the sterilizing solution containing 10% sodium hypochlorite and 0.1% Triton X-100 for 15 min and washed several times by sterilized distilled water. Sterilized seeds were planted on the phytagel-fixed half-strength of Murashige-Skoog medium. Petri dishes were covered with aluminum foil and incubated vertically at 23 C° for 4 d.

Root illumination procedure. For very short illumination times, seedlings were laid on a glass slide and illuminated with the

light source of confocal microscope (using blue filter, 80 µmol m^{-2} s^{-1}). For long time illumination, seedlings placed between slide and cover glass were vertically fixed and illuminated with the halogen lamp (Phillips 20 MR 16, New Jersey, USA) passed through glass filters (Corning #5032) for blue light illumination and without filters for white light illumination.

The visualization of ROS with DAB and PY-1. Diamino benzidine (DAB) was dissolved in water as a stock solution, concentration was at 500 mg/ml. DAB solution (final concentration at 1 mg/ml) was infiltrated into seedlings under darkness with vacuum for 5 min. Seedlings were then illuminated for 60 min by following the procedure described as above. H_2O_2-sensing fluorescent probe, PY-1 ME (Peroxy Yellow-1 Methyl-Ester) was developed and gifted to us by Christopher J. Chang from the University of California, Berkeley, CA, USA.[21] The esterified form of the oxidant-sensing fluorescent probe PY1-ME passes diffusely through the cell membrane and is then de-esterified by the cellular esterase. PY1-ME is trapped inside the cytosolic space and reacts with intracellular H_2O_2 resulting in the observed pattern of fluorescence.[21] Ten millimols of PY1-ME was dissolved in DMSO and stored in -20 C° freezer as stock solution. Dark grown etiolated seedlings were incubated in half-strength MS liquid medium for 30 min to minimize any background of ROS. Seedlings were then transferred into new medium containing 10 µM PY-1 and incubated for 10 min. After treatment of PY-1, seedlings were washed three times in fresh medium. Fluorescence signal in roots were observed under the laser scanning confocal microscope (Fluoview, Olympus, Japan) with excitation at 514 nm and emission at 540 nm. All procedures were accomplished in a dark environment.

Inhibitors and scavengers. Five hundred micromol SHAM (Salicylic hydroxamic acid); peroxidase inhibitor, 10 µM DPI (Diphenylene iodonium); flavoprotein inhibitor including NADPH oxidase and photoreceptors binding flavin molecules, 1mM DDC (Diethyldithiocarbonate); superoxide dismutase inhibitor, 4-OH-TEMPO (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl); superoxide scavenger, 5 mM Tiron (1,2-dihydroxybenzene-3,5-disulfonic acid); superoxide scavenger. For the treatment of inhibitors and scavengers, seedlings were pre-incubated with each chemical for 10 min before the treatment of PY-1. All chemicals used here was purchased from Sigma-Aldrich (St. Louis, MO).

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References

1. Galen C, Rabenold JJ, Liscum E. Light-sensing in roots. Plant Signal Behav 2007; 2:106-8.
2. Galen C, Rabenold JJ, Liscum E. Functional ecology of a blue light photoreceptor: effects of phototropin-1 on root growth enhance drought tolerance in Arabidopsis thaliana. New Phytol 2007; 173:91-9.
3. Rédei GP. Arabidopsis as a genetic tool. Annu Rev Genet 1075; 9:111-27.
4. Koncz C. Dedication: George P. Rédei Arabidopsis geneticist and polymath. Plant Breed Rev 2011; 26:1-33.
5. Miller G, Schlauch K, Tam R, Cortes D, Torres MA, Shulaev V, et al. The plant NADPH oxidase RBOHD mediates rapid systemic signaling in response to diverse stimuli. Sci Signal 2009; 2:ra45.
6. Wong HL, Shimamoto K. Sending ROS on a bullet train. Sci Signal 2009; 2:pe60.
7. Laxmi A, Pan J, Morsy M, Chen R. Light plays an essential role in intracellular distribution of auxin efflux carrier PIN2 in Arabidopsis thaliana. PLoS One 2008; 3:e1510.
8. Baluška F, Mancuso S, Volkmann D, Barlow PW. Root apex transition zone: a signalling-response nexus in the root. Trends Plant Sci 2010; 15:402-8.
9. Baluška F, Mancuso S, Volkmann D, Barlow PW. The "root-brain" hypothesis of Charles and Francis Darwin: Revival after more than 125 years. Plant Signal Behav 2009; 4:1121-7.
10. Potters G, Pasternak TP, Guisez Y, Palme KJ, Jansen MA. Stress-induced morphogenic responses: growing out of trouble? Trends Plant Sci 2007; 12:98-105.
11. Friml J. Subcellular trafficking of PIN auxin efflux carriers in auxin transport. Eur J Cell Biol 2010; 89:231-5.
12. Harir Y, Mittler R. The ROS signaling network of cells. In: del Rio LA, Puppo A, Eds. Reactive oxygen species in plant signaling. Verlag: Springer 2009:165-74.
13. Leivar P, Quail PH. PIFs: pivotal components in a cellular signaling hub. Trends Plant Sci 2011; 16:19-28.
14. Josse EM, Halliday KJ. Skotomorphogenesis: the dark side of light signalling. Curr Biol 2008; 18:R1144-6.
15. Huh SM, Noh EK, Kim HG, Jeon BW, Bae K, Hu HC, et al. Arabidopsis annexins AnnAt1 and AnnAt4 interact with each other and regulate drought and salt stress responses. Plant Cell Physiol 2010; 51:1499-514.
16. Tong H, Leasure CD, Hou X, Yuen G, Briggs W, He ZH. Role of root UV-B sensing in Arabidopsis early seedling development. Proc Natl Acad Sci USA 2008; 105:21039-44.
17. Leasure CD, Tong H, Yuen G, Hou X, Sun X, He ZH. ROOT UV-B SENSITIVE2 acts with ROOT UV-B SENSITIVE1 in a root ultraviolet B-sensing pathway. Plant Physiol 2009; 150:1902-15.
18. Ge L, Peer W, Robert S, Swarup R, Ye S, Prigge M, et al. Arabidopsis ROOT UVB SENSITIVE2/WEAK AUXIN RESPONSE1 is required for polar auxin transport. Plant Cell 2010; 22:1749-61.
19. Leasure CD, Tong HY, Hou XW, Shelton A, Minton M, Esquerra R, et al. Root UV-B sensitive mutants are suppressed by specific mutations in ASPARTATE AMINOTRANSFERASE2 and by exogenous Vitamin B6. Mol Plant 2011; In press.
20. Rizzini L, Favory JJ, Cloix C, Faggionato D, O'Hara A, Kaiserli E, et al. Perception of UV-B by the Arabidopsis UVR8 protein. Science 2011; 332:103-6.
21. Miller EW, Dickinson BC, Chang CJ. Aquaporin-3 mediates hydrogen peroxide uptake to regulate downstream intracellular signaling. Proc Natl Acad Sci USA 2010; 107:15681-6.
22. Dabrowska A, Jacewicz D, Łapińska A, Banecki B, Figarski A, Szkatuła M, et al. Pivotal participation of nitrogen dioxide in L-arginine induced acute necrotizing pancreatitis: protective role of superoxide scavenger 4-OH-TEMPO. Biochem Biophys Res Commun 2005; 326:313-20.
23. Xu XM, Lin H, Maple J, Björkblom B, Alves G, Larsen JP, et al. The Arabidopsis DJ-1a protein confers stress protection through cytosolic SOD activation. J Cell Sci 2010; 123:1644-51.
24. Vitha S, Beneš K, Phillips JP, Gartland KM. Histochemical GUS analysis. In: Gartland KM, Davey MR, Eds. Agrobacterium Protocols. Totowa, NJ: Humana Press 1995:185-93.
25. Tsukagoshi H, Busch W, Benfey PN. Transcriptional regulation of ROS controls transition from proliferation to differentiation in the root. Cell 2010; 143:606-16.
26. Capone R, Tiwari BS, Levine A. Rapid transmission of oxidative and nitrosative stress signals from roots to shoots in Arabidopsis. Plant Physiol Biochem 2004; 42:425-8.
27. Mittler R, Vanderauwera S, Suzuki N, Miller G, Tognetti VB, Vandepoele K, et al. ROS signaling: the new wave? Trends Plant Sci 2011; 16:300-9.