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ORIGINAL PAPER

Reactive oxygen species localization in roots of *Arabidopsis thaliana* seedlings grown under phosphate deficiency

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Abstract *Arabidopsis* plants responding to phosphorus (P) deficiency increase lateral root formation and reduce primary root elongation. In addition the number and length of root hairs increases in response to P deficiency. Here we studied the patterns of radical oxygen species (ROS) in the roots of *Arabidopsis* seedlings cultured on media supplemented with high or low P concentration. We found that P availability affected ROS distribution in the apical part of roots. If plants were grown on high P medium, ROS were located in the root elongation zone and quiescent centre. At low P ROS were absent in the elongation zone, however, their synthesis was detected in the primary root meristem. The proximal part of roots was characterized by ROS production in the lateral root primordia and in elongation zones of young lateral roots irrespective of P concentration in the medium. On the other hand, plants grown at high or low P differed in the pattern of ROS distribution in older lateral roots. At high P, the elongation zone was the primary site of ROS production. At low P, ROS were not detected in the elongation zone. However, they were present in the proximal part of the lateral root meristem. These results suggest that P deficiency affects ROS distribution in distal parts of *Arabidopsis* roots. Under P-sufficiency ROS maximum was observed in the elongation zone, under low P, ROS were not synthesized in this segment of the root, however, they were detected in the apical root meristem.

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Keywords Hydrogen peroxide · Lateral roots · Phosphate availability · Superoxide · Root growth · Root system architecture

Abbreviations

DCF	2′,7′-Dichlorofluorescein
DCFH	2′,7′-Dichlorodihydrofluorescein
NBT	Nitroblue tetrazolium
QC	Quiescent centre
ROS	Radical oxygen species

Introduction

Seedlings of *Arabidopsis thaliana* exhibit remarkable root architectural changes in response to P availability. Plants exposed to low P concentration form a highly branched root system with abundant lateral roots and a short primary root. The reduction in primary root elongation, accompanied by increased root branching allows for the concentration of the root biomass near the soil surface for more efficient nutrient foraging. Lateral root development is followed by the formation of long root hairs and the expression of high-affinity P transporters to optimize P uptake. In contrast, at high P concentration the root system is composed of a long primary root with few lateral roots (Linkohr et al. 2002; Lopéz-Bucio et al. 2002).

The responses of the root system to P deficiency are dependent on changes in cell proliferation. At low P conditions the meristematic activity in the main root is blocked or slowed down and relocated to the sites of lateral root formation. The reduction in primary root growth is due to a

determinate low P-induced root developmental programme that inhibits cell division in the primary root meristem and promotes differentiation within the root tip (Sánchez-Calderón et al. 2005; Lai et al. 2007).

A central role has been proposed for the phytohormone auxin in controlling the development of the root system in response to P deficiency (Hammond et al. 2004). It has been shown that the increased lateral root development in low P conditions is dependent on auxin transport and signalling (López-Bucio et al. 2005). Also the sensitivity of lateral root formation and primary root growth to auxin was found to be increased in P-deprived plants (Lopéz-Bucio et al. 2002). The reduced elongation rate of the primary roots of P-starved plants results from increased auxin accumulation in the root apical meristem (Nacry et al. 2005). On the other hand, López-Bucio et al. (2005) propose that an auxin-independent process is responsible for the primary root growth arrest at low P.

Besides the hormones, certain cellular responses to nutrient deficiency in *Arabidopsis* roots are possibly controlled by reactive oxygen species (ROS). It has been reported that upon deprivation of phosphate, H_2O_2 concentrations in roots increased. The increase in its production was detected several hours after nutrient deprivation. An increase in ROS concentration was also observed in K and N-starved roots. The increase in ROS concentrations is explained by specific up-regulation of different isoforms of a ROS-generating enzyme, NADPH oxidase. It was shown that while one oxidase, *AtrbohC*, was induced by deprivation of all three nutrients, it was expressed most abundantly and earlier after K deprivation. The other enzyme, *AtrbohA*, was mainly induced under P deprivation (Shin et al. 2005).

The increase in ROS production may play an important role in modulating the induction of some of the genes expressed in response to nutrient deficiency. It was observed that treatments with diphenylene iodonium (DPI), an inhibitor of NADPH oxidase, eliminated or greatly reduced the increased expression of K^+ transporter genes, transcription factor WRKY9 and two peroxidases that were normally up-regulated after K deprivation. The expression of the aforementioned genes was also eliminated in NADPH oxidase mutant *rhd2*. Expression of these genes could be restored by H_2O_2 or by H_2O_2 in combination with K^+ deprivation (Shin and Schachtman 2004).

In this study we tested the distribution of ROS in different parts of the roots of *Arabidopsis* seedlings grown on media supplemented with high or low phosphate concentration. ROS were localized in the apices of the primary roots, lateral root primordia and lateral roots of seedlings grown on the media supplemented with a sufficient (1 mM NaH_2PO_4) or deficient phosphate (0.001 mM NaH_2PO_4) concentration.

Materials and methods

Plant material and growth conditions

Seeds of *Arabidopsis thaliana* (ecotype Columbia) were soaked in sterile distilled water for 30 min and surface sterilized with 95% (v/v) ethanol for 5 min and with 20% (v/v) bleach for 7 min. Then seeds were washed several times in sterile water and sown onto the surface of the culture media in Petri dishes. Two media, differing in their phosphate concentration, were applied; the first one contained 0.001 mM NaH_2PO_4, and the second one 1 mM NaH_2PO_4 in the Murashige and Skoog (1962) medium modified according to Lopéz-Bucio et al. (2002). Before the culture was started, the dishes were placed in darkness at 4°C for 48 h to promote and synchronize germination (Lopéz-Bucio et al. 2002). Germination percentages were 81 ± 7 and 84 ± 11 at high P and low P medium, respectively. Seedlings were grown at 25°C with a photoperiod of 16 h of light, 8 h of darkness with standard irradiation 431 μmol m^{-2} s^{-1}, provided by Osram 30 W/11-860 "Daylight" fluorescent tubes (Osram, Berlin, Germany). The features of "low" and "high" P root architecture were clearly discernible after 10 days of culture. Then the seedlings were destined for analysis.

Reactive oxygen species localization

Two staining methods were applied to study the patterns of ROS accumulation in roots. The first one made use of 2′,7′-dichlorodihydrofluorescein (DCFH) diacetate which exhibits selectivity for H_2O_2; nevertheless the assay provides an integral assay for several ROS because it is likely that in vivo, other radical species are quickly converted to the more stable H_2O_2 (Rodriguez et al. 2002). DCFH-diacetate can cross the plasma membrane and after being deacetylated by endogenous esterase, liberates DCFH in the cytoplasm, where it is oxidized in the reaction with ROS to highly fluorescent 2′,7′-dichlorofluorescein (DCF; Schopfer et al. 2001). In order to load the dye into the cells the roots were incubated for 15 min in 50 mM phosphate buffer (pH 7.5) containing 50 μM DCFH-diacetate. After that the roots were rinsed with the phosphate buffer and imaged in an Eclipse (Nicon) confocal microscope using 488 nm excitation and 525 nm emission spectra (Zhang et al. 2001). Optical sections were collected with a *z* focus increment of 5 μm. To test the assay for the non-specific fluorescence, control seedlings were, prior to DCFH-assay, pre-treated for 60 min with 10 mM KI which is a known H_2O_2 scavenger (Dunand et al. 2007).

The second method was specific for superoxide radical ($O_2^{\bullet -}$), which was localized in roots according to Mellersh et al. (2002). The sites of superoxide radical production in

roots were localized by incubating the seedlings in 0.005% NBT dissolved in 100 mM phosphate buffer, pH 7.5 for 10 min. After that time, incubation was interrupted by replacing the NBT solution with phosphate buffer. NBT forms an insoluble formazan product upon reduction by $O_2^{\bullet -}$ (Bielski et al. 1980). The sites of superoxide radical accumulation stained dark blue. To verify the specificity of staining, control seedling were incubated for 60 min in a water solution of an antioxidant propyl gallate (10 mM), before the NBT staining was performed.

Each experiment was repeated three times. The photographs show the representative plant chosen from at least 25 plants analyzed in each experiment.

Results and discussion

Root architecture under sufficient and deficient phosphate availability

The essential parameters characterizing root system architecture of plants used in this study are enclosed in Table 1. The seedlings of *Arabidopsis thaliana* grown 10 days on the media supplemented with 1 mM phosphate were characterized by long primary roots and few lateral roots (Fig. 1a). In contrast, seedlings exposed to 0.001 mM phosphate formed a short primary root and numerous lateral roots (Fig. 1b). These observations are in agreement with those of other authors who reported that the culture of seedlings at low P concentration (1–100 μM), resulted in primary root growth inhibition and enhanced production of lateral roots (Williamson et al. 2001; Lopéz-Bucio et al. 2002). In our studies, the features of "low" and "high" P root architecture were clearly discernible just 10 days after germination (Fig. 1; Table 1). These findings are similar to the results of Williamson et al. (2001) and López-Bucio et al. (2005), who report that RSA parameters are affected by P starvation at an early stage of seedling development (i.e., 5–6 days after germination). In contrast, in the experiments of Nacry et al. (2005) some effects of low P (especially primary root growth inhibition) were much more delayed and manifested themselves 13 days after planting.

ROS distribution in apical parts of roots of *Arabidopsis* seedlings grown in the presence of sufficient or deficient phosphate concentration

The apex of the primary root was reported to have two areas of ROS production: the quiescent centre (QC) and the elongation zone (Jiang et al. 2003; Liszkay et al. 2004). The oxidative environment in the QC was found to be important for maintaining the low cell division rate in the QC (Jiang et al. 2003). ROS production in the elongation zone of roots represents a common feature of seed plants and is possibly related to the promotion of the increase in cell wall extensibility within this part of the root (Liszkay et al. 2004). Cell wall loosening is thought to result form an

Table 1 Effect of phosphate availability on selected root system architecture parameters of *Arabidopsis* seedlings

0.001 mM NaH_2PO_4				1 mM NaH_2PO_4			
Primary root length (mm)	Lateral root length (mm)	Lateral root number	Lateral root density	Primary root length (mm)	Lateral root length (mm)	Lateral root number	Lateral root density
31.2 ± 3.5	18.1 ± 2.7	7.5 ± 2.5	2.7 ± 1.1	95.4 ± 6.2	4.5 ± 2.9	4.6 ± 1.8	0.5 ± 0.1

Primary root length, lateral root length, lateral root number and lateral root density were determined after 10 days of culture on media supplemented with 0.001 mM or 1 mM NaH_2PO_4. Lateral root density was calculated to normalize for the effects of P availability on root length by dividing the number of lateral roots by the length of the primary roots. Values represent the mean of at least 25 seedlings ± SD

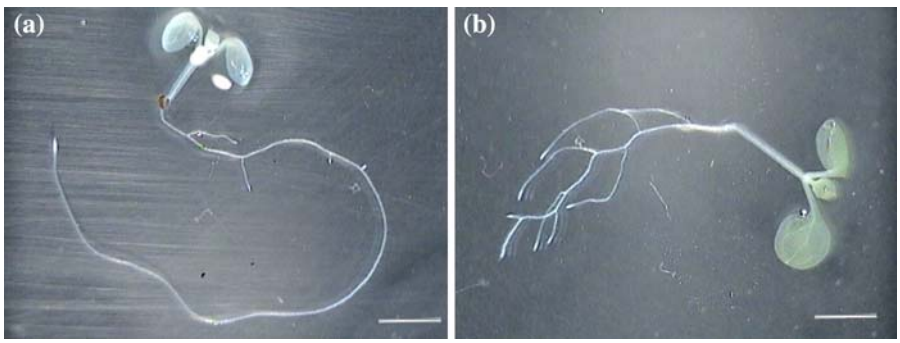

Fig. 1 Seedlings of *Arabidopsis thaliana* cultured 10 days on the 0.1 MS medium containing 1 mM (**a**) or 0.001 mM phosphate (**b**). *Scale bar* = 1 cm

oxidative scission of cell wall polysaccharides due to hydroxyl radical ($^{\bullet}OH$) production in the apoplast. $^{\bullet}OH$ is formed in the reaction of NAD(P)H oxidation catalyzed by apoplastic peroxidases with $O_2^{\bullet-}$ and H_2O_2 serving as intermediates (Liszkay et al. 2003, 2004). $^{\bullet}OH$ can also be generated nonenzymatically, in the presence of H_2O_2, $O_2^{\bullet-}$, ascorbate and Cu, which are all usually present in the apoplast (Fry 1998, Green and Fry 2005).

In our study, the analysis of DCF fluorescence revealed that a typical pattern of H_2O_2 distribution in the root apex with the local maxima in the QC and elongation zone was clearly visible in roots of seedlings grown in the presence of high (1 mM) phosphate concentration. The area localized between the QC and the elongation zone was also marked by H_2O_2 production. They were present in strands of cells of differentiating pericycle/endoderm layer (Fig. 2a, a′). The H_2O_2 distribution pattern in root apices was changed if plants were grown on medium with 0.001 mM phosphate. DCF fluorescence had its maximum in the QC and in lateral parts of the proximal root meristem. H_2O_2 concentration gradually decreased towards the base of the root and no distinct fluorescence maximum was detected within the elongation zone (Fig. 2b, b′). DCF fluorescence in roots was efficiently reduced by KI, in accordance with its scavenging effect on H_2O_2 (Fig. 2a″, b″).

Histochemical detection of superoxide radical was accomplished with nitroblue tetrazolium chloride (NBT). Formazan formation, resulting for NBT reduction, was not observed if seedlings were pre-treated with an antioxidant propyl gallate (Fig. 3a″, b″). This finding confirms that an assay is ROS-specific. Roots of seedlings cultured in the presence of sufficient phosphate conditions i.e., 1 mM phosphate produced $O_2^{\bullet-}$ in the QC, elongation zone and at the border between cortex and vascular tissue (Fig. 3a′). In contrast, roots of seedlings grown with 0.001 mM phosphate accumulated $O_2^{\bullet-}$ in the QC and proximal meristem, while the elongation zone was $O_2^{\bullet-}$ free (Fig. 3b′). We observed that the patterns of NBT staining were comparable with the results of the DCFH assay. On the other hand, Dunand et al. (2007) detected a difference in the patterns of $O_2^{\bullet-}$ and H_2O_2 accumulation in *Arabidopsis* roots. The authors reported that the elongation zone and to a lesser extent were rich in $O_2^{\bullet-}$, while H_2O_2 predominated in a differentiation zone (Dunand et al. 2007).

Shin and Schachtman (2004) and Shin et al. (2005) reported that a common response during N-, P- and K-deficient conditions was the increase in H_2O_2 concentration in distal parts of roots. ROS increased in response to P deprivation in the root cortex, whereas H_2O_2 increased in the epidermis after N and K deficiency. The authors reported that nutrient deficiency-induced ROS production

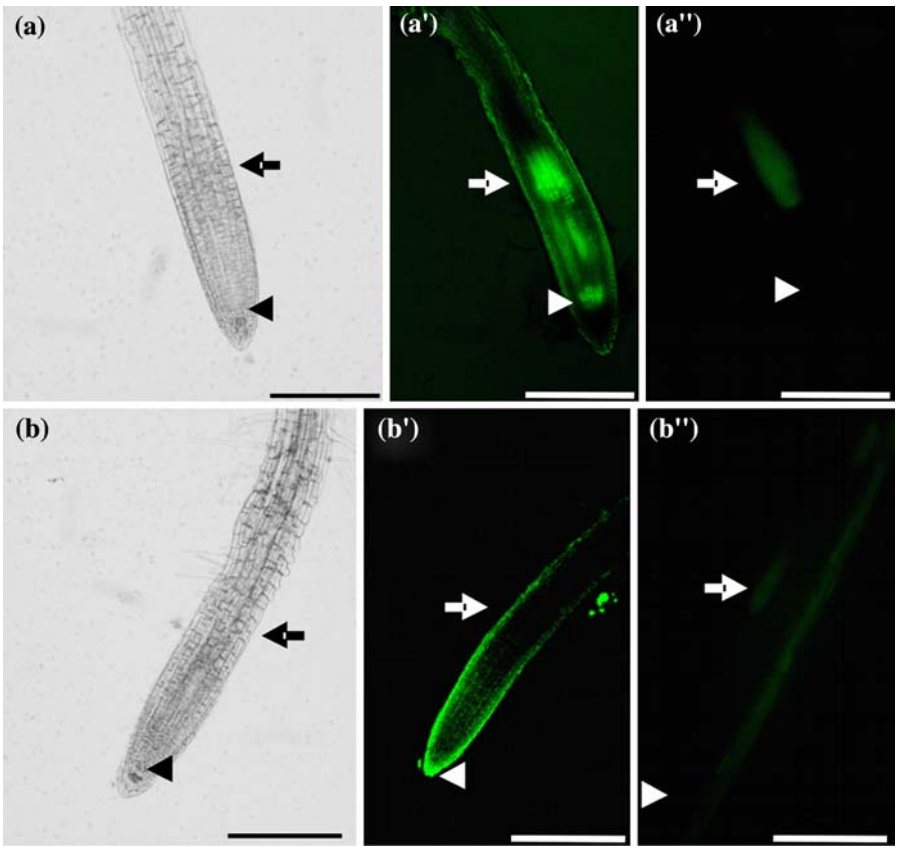

Fig. 2 DCF fluorescence indicating the sites of H_2O_2 production in root apices of the seedlings of *Arabidopsis thaliana*. Plants were cultured 10 days at 1 mM (**a**, **a**′, **a**″) or 0.001 mM phosphate (**b**, **b**′, **b**″). Photographs show bright field images of root apices (**a**, **b**), DCF fluorescence (**a**′, **b**′) or DCF fluorescence in roots pre-treated with 10 mM KI (**a**″, **b**″). *Arrows* indicate root elongation zone, *arrowheads* indicate apical root meristem. *Scale bar* = 1 mm

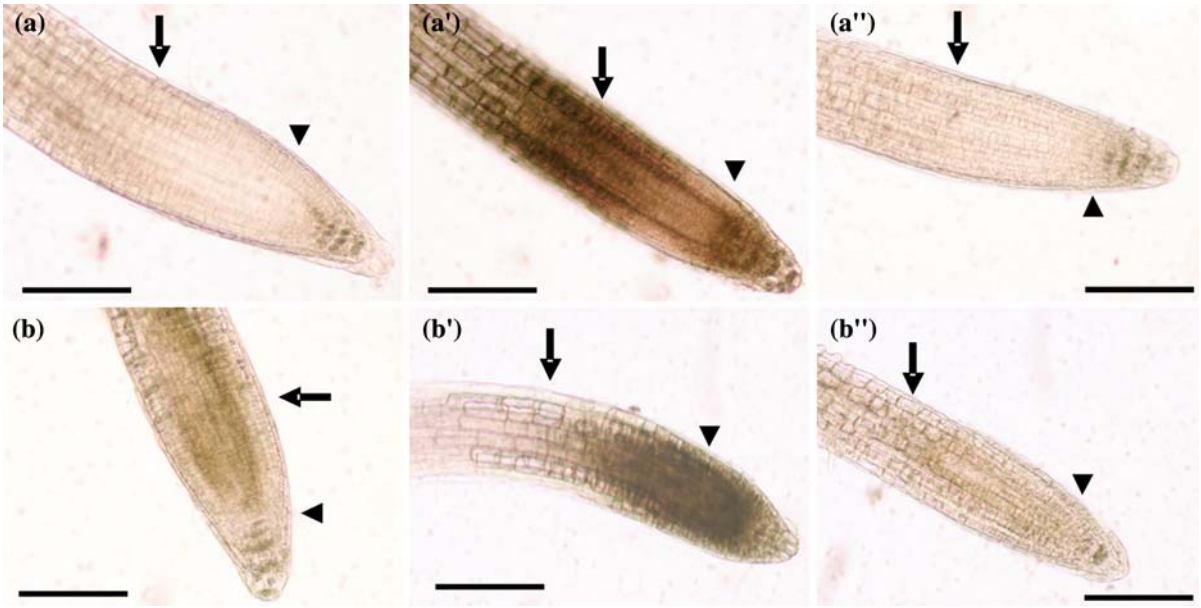

Fig. 3 NBT staining indicating the production of superoxide radical in root apices of the seedlings of *Arabidopsis thaliana*. Plants were cultured 10 days at 1 mM (**a**, **a**′) or 0.001 mM phosphate (**b**, **b**′). Photographs show root apices stained with NBT (**a**′, **b**′), no-stain controls (**a**, **b**) or controls pre-treated with 10 mM propyl gallate before NBT staining (**a**″, **b**″). *Arrows* indicate root elongation zone, *arrowheads* indicate apical root meristem. *Scale bar* = 1 mm

occurred in the region just behind the elongation zone which is engaged in nutrient uptake while no changes were reported for the meristematic part of the root tip (Shin and Schachtman 2004; Shin et al. 2005). In contrast to these data, we observed a P deficiency-dependent alteration in ROS distribution mainly in the meristem and elongation zone. However, it should be kept in mind that an increase in ROS level in the part of the root engaged in nutrient uptake, reported by Shin et al. (2005), was measured after 6–30 h of P deprivation. Therefore, it represents an early response to nutrient deficiency (Shin et al. 2005). Moreover, they used 5-day-old plants to localize the generation of ROS in the roots. In contrast to their approach, in our study, the imaging of ROS was conducted after 10 days of culture under P-deficient conditions and was performed using the fluorescent probe different from that which was applied by Shin et al. (2005).

It was reported that low P treatment affects two components that mediate root elongation; cell divisions in the root apical meristem and cell elongation in the root elongation zone. Phosphate deficiency induces the developmental programme that inhibits cell division and promotes cell differentiation in the primary root meristem and reduces cell elongation in the elongation zone (Sánchez-Calderón et al. 2005). Our data suggest that these changes are accompanied by the elimination of the pattern of ROS distribution typical for the growing root including the absence of ROS in the elongation zone. ROS production in this segment of the root is considered as an important factor accelerating root growth (Liszkay et al. 2004).

The mechanism responsible for the change in ROS localization in root tips needs further studies. It has been reported that ROS production in the root meristem is determined by the pattern of auxin distribution (Joo et al. 2001; Jiang et al. 2003). Therefore an alteration in ROS localization in the root tip of a P-deficient plant may be related to the changes in auxin localization in the root tips of P-deficient plants. However, the data on the effect of low P level on auxin localization in *Arabidopsis* root tips are not consistent. After 10 days of culture at low P, the auxin level in the root meristem was reported to be strongly reduced (López-Bucio et al. 2005). In contrast, a 14-day-long culture with P-deficiency resulted in an auxin over-accumulation in the primary root meristem (Nacry et al. 2005). At high P, the hormone has its maximum in the QC and columella (López-Bucio et al. 2005; Nacry et al. 2005).

The localization of ROS in lateral roots, lateral root primordia and root hairs

The analysis of the distribution of DCF fluorescence in the proximal part of the roots revealed that H_2O_2 was preferentially accumulated in the lateral root primordia (Fig. 4). H_2O_2 accumulation in lateral root primordia was observed in roots of plants grown under both phosphate regimes. In Fig. 4, we show H_2O_2 localization in LR primordia and

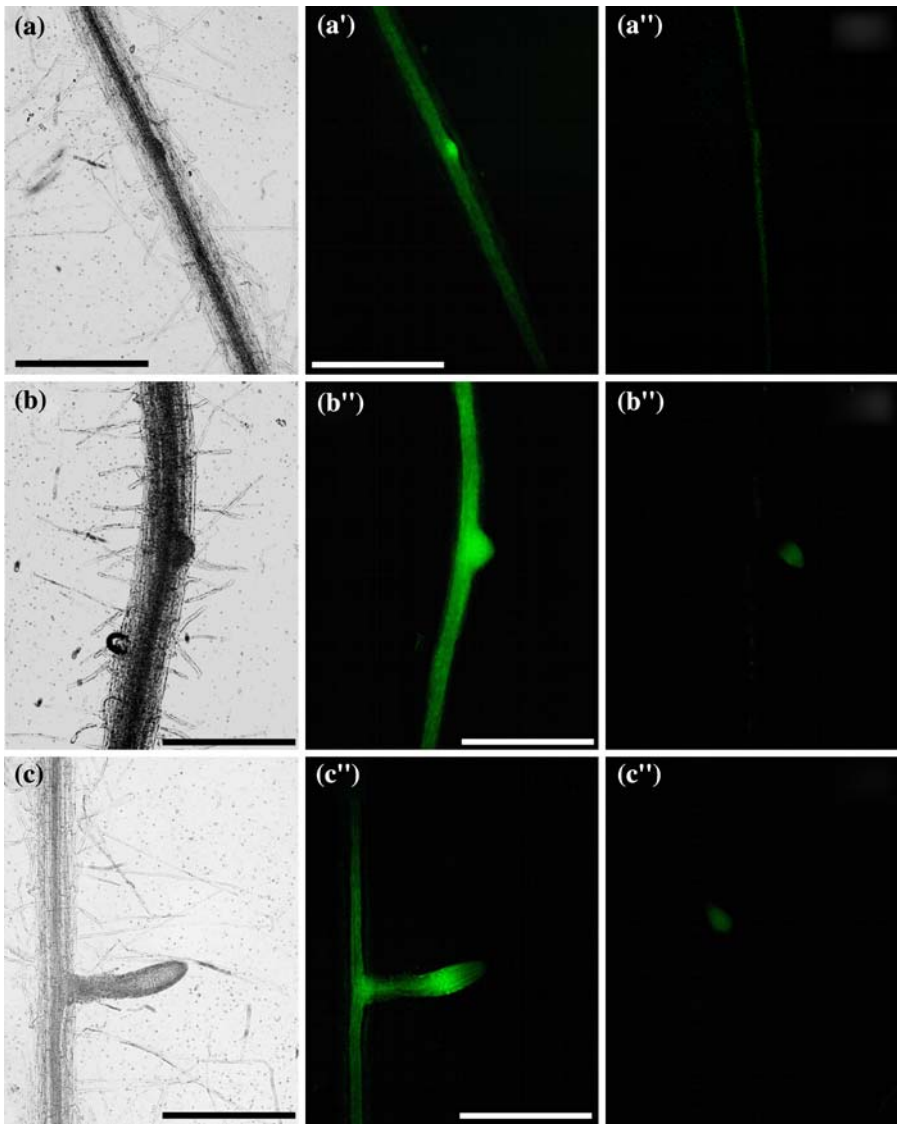

Fig. 4 DCF fluorescence indicating the sites of H_2O_2 production in the lateral root primordia and in young lateral roots. *Arabidopsis* seedlings were cultured 10 days at 1 mM phosphate. Photographs show bright field micrographs (**a**) and DCF fluorescence (**a′**) of a primordium at an initial stage of development, bright field micrographs (**b**) and DCF fluorescence (**b′**) of an organized primordium growing across the cortical tissues of the primary root, bright field micrographs (**c**) and DCF fluorescence (**c′**) in a newly formed lateral root and DCF fluorescence in a primordium at an initial stage of development (**a″**), an organized primordium (**b″**) and a young lateral root (**c″**) after 60 min-long incubation in a 10 mM KI. *Scale bar* = 1 mm

young lateral roots formed at high P. High DCF fluorescence characterized both the stage of primordium initiation (Fig. 4a, a′) and its further development (Fig. 4b, b′). After the primordium developed into a young lateral root the maximum of DCF fluorescence became restricted to the elongation zone; however, H_2O_2 was also produced in cells localized at the base of the lateral root (Fig. 4c, c′).

The presence of ROS in lateral root primordia may possibly be related to auxin, which is known to stimulate their production (Joo et al. 2001) and is accumulated within these structures (López-Bucio et al. 2005; Nacry et al. 2005). This idea, however, needs further testing. The putative function of ROS in lateral root primordia is not clear. Some data point to the presence of the cellular redox systems within these structures. The gene coding for the cytosolic ascorbate peroxidase is selectively expressed in lateral root primordia. This enzyme may be responsible for the regulation of H_2O_2 level in lateral root primordia (Gadea et al. 1999). Finding that nitric oxide mediates an auxin-dependent pathway of lateral root primordia initiation suggests that redox agents are possibly engaged in their formation (Correa-Aragunde et al. 2006).

Phosphate availability affected the pattern of H_2O_2 accumulation in apical parts of older lateral roots. At sufficient (1 mM) phosphate concentration H_2O_2 accumulated in the elongation zone of the growing root (Fig. 5). At low P, a visibly lower DCF fluorescence was observed when compared to high P conditions (Fig. 5a′ vs. b′). Moreover, at P deficiency, H_2O_2 accumulated in a proximal part of the root apical meristem while no H_2O_2 were detected in the elongation zone. At low P, H_2O_2 production was restricted to the internal tissues while H_2O_2 was absent in the epidermis of the root apex (Figs. 5b′, 6b′). It is also noteworthy that the proximal parts of the primary roots of

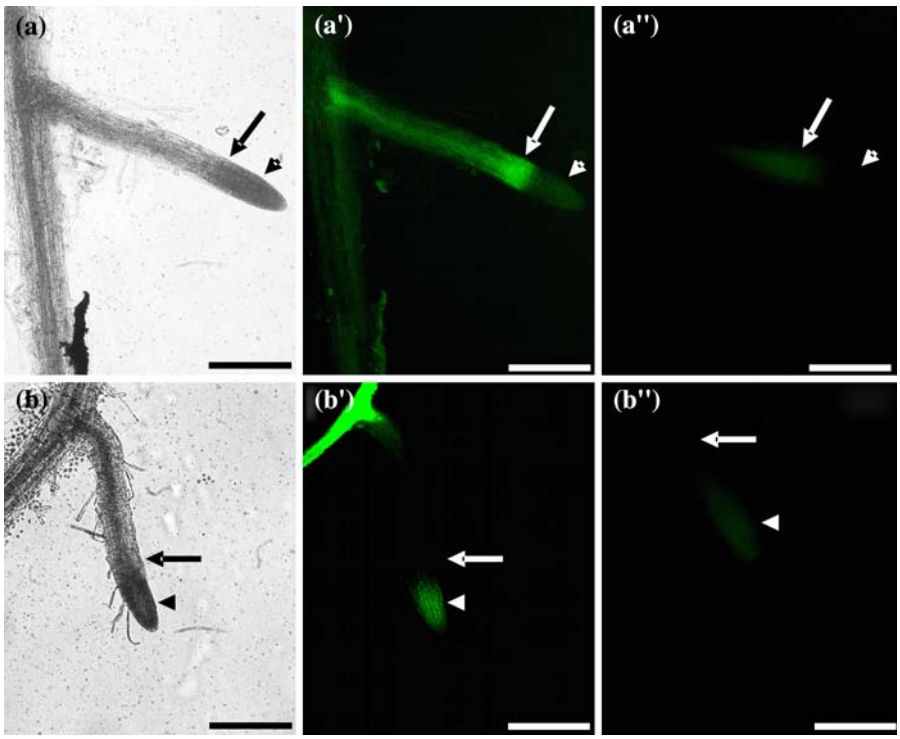

Fig. 5 DCF fluorescence indicating the sites of H_2O_2 production in lateral roots of the seedlings of *Arabidopsis thaliana*. Plants were cultured 10 days at 1 mM (**a**, **a′**, **a″**) or 0.001 mM (**b**, **b′**, **b″**) phosphate. Photographs show bright field images (**a**, **b**), DCF fluorescence (**a′**, **b′**) or DCF fluorescence in lateral roots after incubation in 10 mM KI (**a″**, **b″**). *Arrows* indicate root elongation zone, *arrowheads* indicate apical root meristem. *Scale bar* = 1 mm

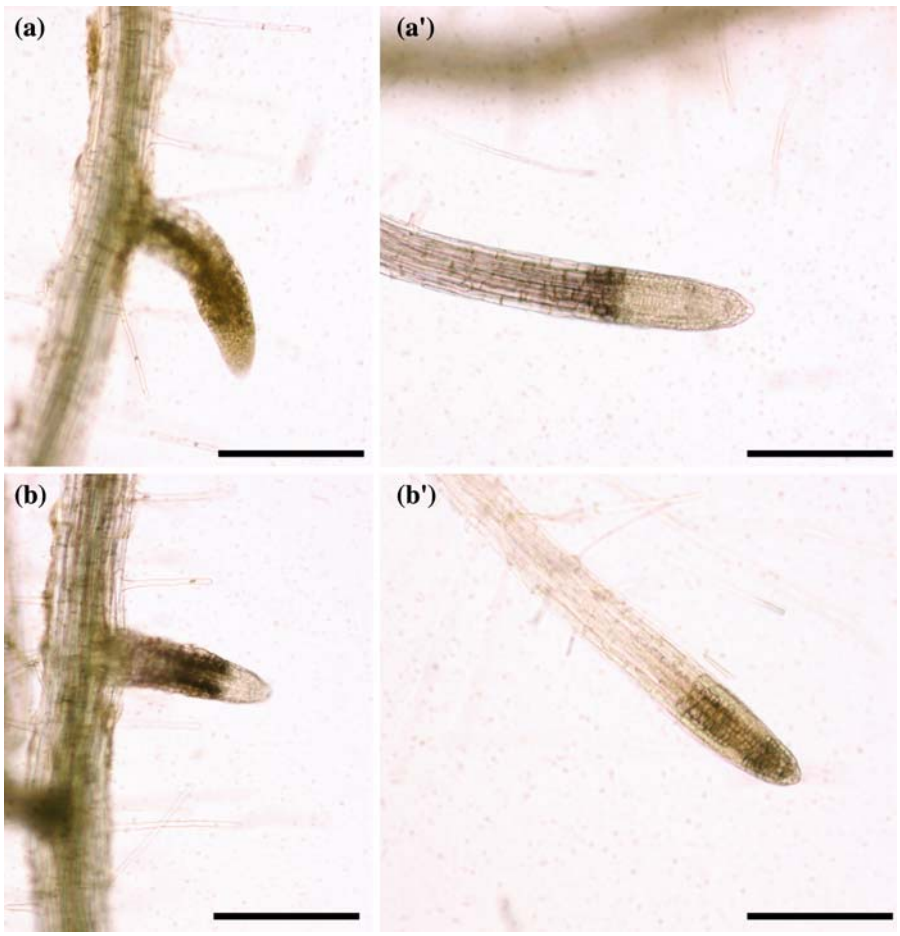

Fig. 6 NBT staining in the apices of the young (**a**) and old (**a′**) lateral roots of *Arabidopsis* seedlings cultured 10 days on the medium supplemented with 1 mM phosphate and in young (**b**) and old (**b′**) lateral roots of *Arabidopsis* seedlings cultured 10 days in the presence of 0.001 mM phosphate. *Scale bar* = 1 mm

phosphate deficient plants accumulated H_2O_2 in the central vein (Figs. 5b′, 7a). If plants were grown in phosphate sufficient conditions H_2O_2 accumulation in the vascular tissues was not observed (Figs. 5a′, 7b).

The maximum of NBT staining was observed in the elongation zone of young lateral roots at both high (Fig. 6a) and low (Fig. 6b) phosphate concentrations. A different situation was observed if older lateral roots were considered. Older lateral roots of phosphate sufficient plants accumulated $O_2^{\bullet-}$ only in the elongation zone while the meristematic part of the root apex was $O_2^{\bullet-}$-free (Fig. 6a′). If organs belonged to plants grown under phosphate deficiency, $O_2^{\bullet-}$ was produced in the proximal part of the apical root meristem (Fig. 6b′). Sánchez-Calderón et al. (2005) have shown that mature lateral roots (similar to primary roots) react with the decline in the mitotic activity in the meristem and subsequent growth arrest to low P conditions. This raises the question of the possible role of the change in the ROS localization in the root tip in a determinate growth programme induced in

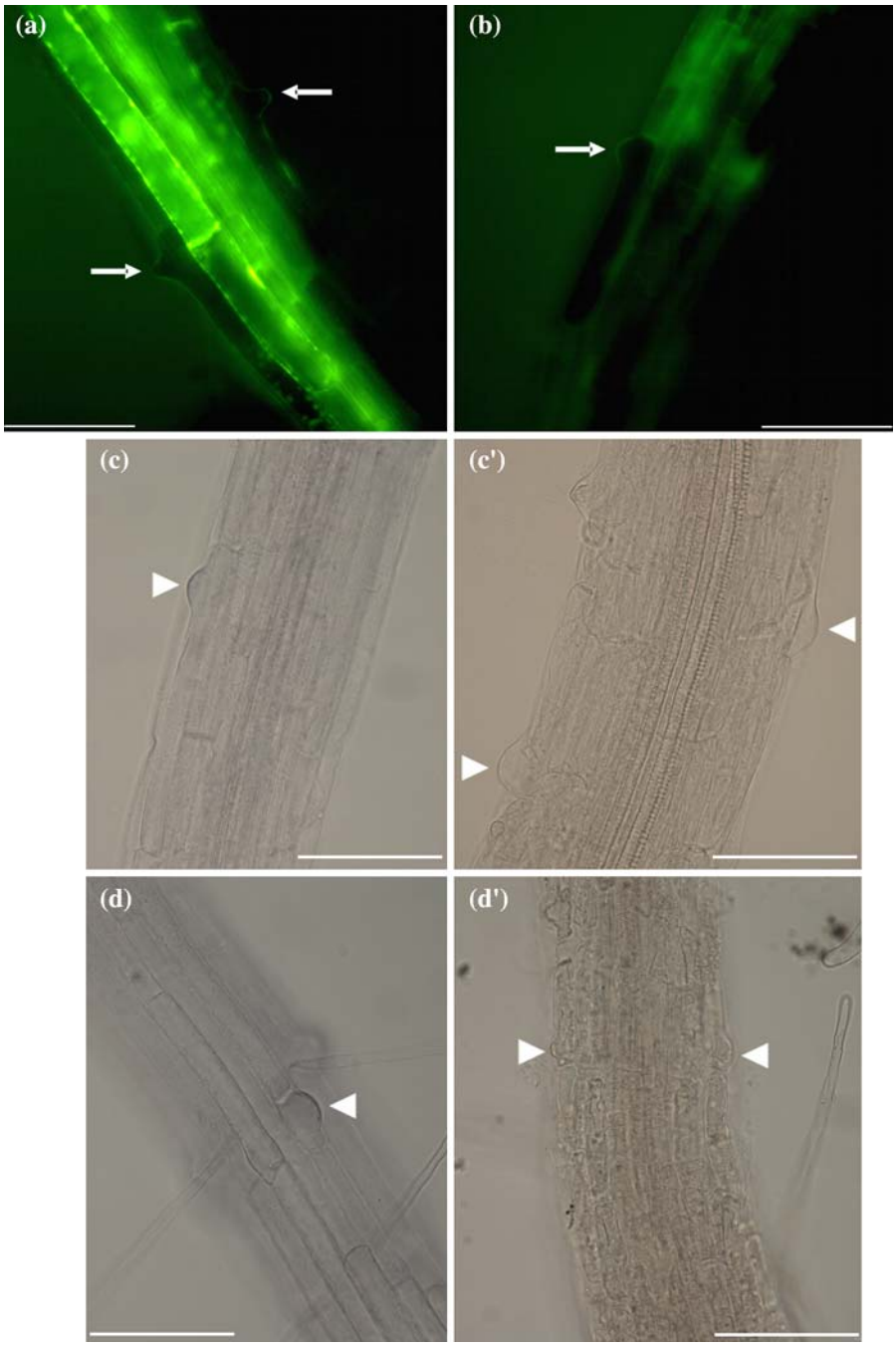

Fig. 7 The sites of H_2O_2 (**a**, **b**) and $O_2^{\bullet-}$ production (**c**, **d**) in the forming root hairs. H_2O_2 was visualized by DCF fluorescence in differentiation zones of roots of *Arabidopsis* seedlings grown 10 days on the medium with low (**a**) or high (**b**) phosphate concentration. Plants grown at low P exhibited a strong DCF fluorescence in central vein (**a**). If plants were grown at high P, H_2O_2 accumulation in the vascular tissues was not observed (**b**). Bright field images show $O_2^{\bullet-}$ distribution visualized by NBT staining in the in differentiation zones of roots of *Arabidopsis* seedlings grown 10 days on the medium with low (**c**) or high (**d**) phosphate concentration. (**c**′) and (**d**′) show roots of control plants grown at low (**c**′) or high P (**d**′) which prior to NBT staining were preincubated with a ROS-scavenging agent, propyl gallate. *Scale bar* = 50 μm

lateral roots at P deficiency. However, this hypothesis needs further studies.

DCF fluorescence and NBT staining was also detected in the forming root hairs in the differentiation zones of roots (Fig. 7). This finding is consistent with previous reports which stressed the role of ROS in the root hair tip growth (Foreman et al. 2003). ROS were produced in the trichoblasts by plants cultured under both phosphate concentrations. We observed that H_2O_2 and $O_2^{\bullet-}$ usually accumulated preferentially at the hair dome and not at the surrounding parts of the cell wall of the trichoblast (Fig. 7a, a′). However, DCF fluorescence was occasionally observed also in the cell wall parts which were not directly engaged in the hair dome formation (Fig. 7a).

In conclusion, we report that a 10-day-long low P treatment affects ROS distribution in the tips of the primary root and the mature lateral roots of *Arabidopsis* seedlings. The most significant changes that occur during culture at P-deficiency involve the depletion of ROS from the root elongation zone and ROS accumulation in the proximal part of the apical root meristem.

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