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ARTICLE

Nitric oxide (NO) generation during vegetative/generative transition of the apical meristem in wheat

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ABSTRACT The phase transition from vegetative to reproductive development of the shoot apical meristem (SAM) is a critical event during the life cycle of seed-propagated plants. Nitric oxide (NO) as a general plant signal plays a role during growth and development and under biotic and abiotic stress conditions. In this study, apical meristems of spring wheat (*Triticum aestivum* L. cv. Thaifun) were isolated during their development and NO was detected by DAF-FM using a fluorescent microscope. To test the NO specificity of DAF-FM, *in vivo* and *in vitro* experiments were also carried out. In vegetative apices NO level was very low and it significantly enhanced during transition (in the „double ridge" phase). After transition, NO level decreased and proved to be low in the generative phase (11th-13th week). Our results show that DAF-FM is a suitable and specific indicator of NO. The significant increase in NO level during the vegetative/generative transition of wheat SAM suggests its regulatory function.
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KEY WORDS

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For reproductive success, timing of apex transition from vegetative to reproductive state is essential. This process is controlled by environmental (e.g. temperature, day length) and endogenous factors (e.g. age, developmental phase) and signals (Galiba et al. 2009). In the rich signal transduction network between the endogenous and environmental stimuli, signal molecules make contact between the components to ensure the phasing of signals. This system is able to maximise the reproductive success of a plant, by ensuring that flower development occurs only under favourable conditions. Nitric oxide (NO) is an inorganic free radical which acts during growth and development and abiotic/biotic stress responses in plants (Kolbert et al. 2008; Lehotai et al. 2011). The participation of NO in the signal transduction of floral determination was discovered by He et al. (2004). Nitric oxide treatment delays vegetative/generative transition and *nox1* (nitric oxide overproducer 1) *Arabidospsis* mutant shows a late-flowering phenotype. Exogenous NO represses the gene expression of the important determinant in flower initiation, LEAFY (LFY) and promotes the accumulation of the key repressor of flowering, the MADS box transcription factor, FLOWERING LOCUS C (FLC). The expression of the circadian clock-regulated CONSTANS (CO) is also decreased by NO. Other NO-modulated genes, mainly NO-inducible, in lesser extent repressible were found as related to abiotic and biotic stresses, like WRKYs, EREBPs, DREB1 and DREB2, etc. (Palmieri et al. 2008). S-nitrosylation is a NO-specific posttranslation modification, which influences the activity of transcription factors, thus regulates gene expression (Astier et al. 2011). In this work we hypothesize that NO plays a role in vegetative/generative transition of the apex, therefore we investigated the possible changes of NO levels in shoot apical meristems of spring wheat, which reflect the involvement of this signal molecule in meristem transition.

Materials and methods

Plant material, SAM isolation

During the experiments wheat plants (*Triticum aestivum* L. cv. Thaifun, spring wheat) were used. The seeds were surface sterilized with 5% (v/v) sodium hypochlorite for 10 min, rinsed and imbibed for 60 min in running water. Seeds were germinated in Petri dishes between moisture filter papers at 25°C for 2 days then they were placed to 4°C for 24 h. After two additional days of germination at room temperature, the seedlings (2-3 cm) were planted into soil (five plants/pot). Plants were grown under controlled conditions in greenhouse at photo flux density of 150 μmol m^{-2} s^{-1} (12/12 day/night period) at a relative humidity of 55-60%, and 25 ± 2°C temperature for 13 weeks.

Shoot apical meristems were isolated weekly from the plants under Zeiss Axioskope 2000-C (Carl Zeiss, Jena, Germany) stereomicroscope using a fine needle and digital images were taken. The isolated meristems were kept in Tris-HCl buffer (pH 7.2) until the microscopic examination.

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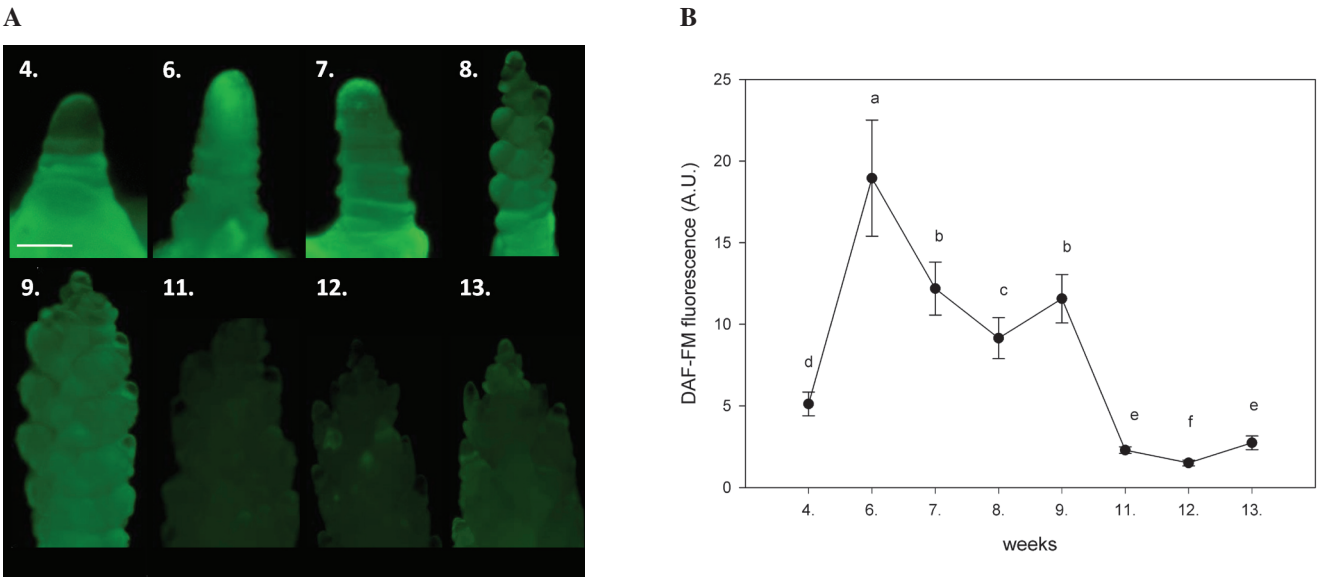

Figure 1. Representative fluorescent microscopic images of DAF-FM-stained apical meristems isolated from 4-,6-,7-,8-,9-,11-,12-,13-week-old wheat plants (A) Bar=1mm. Intensity of nitric oxide-specific DAF-FM fluorescence in wheat apical meristems (B). Values are means of 4-5 plants ±SE. Different letters indicate significant differences (P<0.05) according to Duncan's test.

Verification of the NO-specificity of DAF-FM *in vivo* and *in vitro*

Isolated AMs were incubated in NO donor and/or scavenger solutions (100 µM sodium nitroprusside, SNP; 100 µM S-nitroso-N-penicillamine, SNAP and/or 100 µM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-l-oxyl-3-oxide, cPTIO) at 150 µmol m^{-2} s^{-1} light intensity for 2 hours then they were dyed with 10 µM 4-amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM DA in Tris-HCl buffer, pH 7.2) for 30 min in darkness at room temperature. Samples were washed with the buffer solution 2 times within 30 min and were placed on microscopic slides.

For the *in vitro* experiments the fluorescent DAF-FM molecule was obtained by the alkali hydrolysis of DAF-FM DA according to Balcerczyk et al. (2005). Fluorescent intensities were measured by a fluorescent spectrophotometer (Hitachi F-4500, Hitachi Ltd., Tokyo, Japan). Different concentrations of nitric oxide donor (50, 500, 1000 µM SNP), scavenger (200 µM cPTIO) and hydrogen peroxide (5, 50, 100 µM H_2O_2) solutions were prepared and 2 µM DAF-FM were added. Excitation wavelength was set at 490 nm. The intensity of DAF-FM fluorescence emission was measured at 515 nm.

Fluorescent microscopy and image analysis

Wheat apical meristems labelled with DAF-FM fluorophore was investigated under Zeiss Axiowert 200M invert microscope (Carl Zeiss, Jena, Germany) equipped with a high resolution digital camera (Axiocam MR, HQ CCD, Carl Zeiss, Jena, Germany) and filter set 10 (exc.: 450-490 nm, em.: 515-565 nm). The intensity of NO-dependent fluorescence was measured on digital images within area of circles with 60 µm radii with the help of Axiovision Rel. 4.8 software. The radii of circles were not modified during the experiments.

Statistical analysis

Results are expressed as mean ± SE. Statistical analysis was performed applying SigmaStat 11. software using Duncan's test (P<0.05) and Student's *t*-test (*P≤0.05, **P≤0.01, ***P≤0.001). In each treatment at least 4-5 samples were measured.

Results and Discussion

DAF-FM is a suitable indicator of NO level changes

In NO donor-treated (SNP and SNAP) apices significantly higher DAF fluorescence was measured compared to control, while addition of the NO scavenger decreased the fluorescence. Sodium nitroprusside resulted in higher DAF fluorescence than SNAP, and cPTIO caused ~50% inhibition of the fluorescence intensities. Similar results were obtained by *in vitro* measurements, where SNP significantly increased the intensities of DAF fluorescence and its effect proved to be concentration-dependent. Hydrogen peroxide (5, 50, 100 µM) caused no increase in fluorescent intensities. Our data suggest that the fluorescence intensity of DAF-FM alters according to the endogenous NO content of the apical meristem and the fluorescent emission is independent from the presence of

H_2O_2. Based on these, DAF-FM is considered to be a suitable, NO-specific fluorophore.

Two peaks of NO generation during apical meristem development

The developmental stages (vegetative, „double ridge [DR]", generative) were determined weekly in isolated SAMs. In four-week-old wheat vegetative apices could be found, while the „double ridge" phase was determined in the 6th week. From the 7th week generative apices could be isolated. In the vegetative stage apical meristems showed low NO fluorescence, which significantly increased (4-fold) in DR phase. During the following weeks (7th and 8th weeks, generative apices) NO levels decreased, while in the 9th week a slight NO formation was detected in AMs. From the 11th week NO fluorescence decreased under the level detected in the vegetative stage (Fig. 1). In this work it was shown the first time, that vegetative/reproductive transition of the wheat SAM is accompanied by a notable NO formation, which suggests the possible regulatory role of NO in this developmental process. External and internal signals may regulate the endogenous NO content, which then relays these signals to the transcription regulatory system that controls the vegetative/generative transition of SAM (He et al. 2004). The accumulated NO in the "double ridge"-phase wheat SAM may act as a repressor of flowering through decreasing the expression of CO and/or LFY genes and inducing FLC expression.

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