Zeatin- and Polyamine-Induced Nitric Oxide Biosynthesis in *Arabidopsis thaliana* seedlings and its Role in Signal Transduction

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ABSTRACT

The effect of zeatin and different polyamines (PAs) on NO (nitric oxide) biosynthesis in *Arabidopsis* seedlings and *Nicotiana tabacum* cells was examined in this study. Using a cell-impermeable NO-specific fluorescent dye DAR-4M (diaminorhodamine-4M), the release of NO into the incubation medium was measured. NO production was stimulated by zeatin in both *Arabidopsis* wild type and nitrate reductase (NR) deficient *nia1 nia2* double mutant seedlings. NO production was dependent on zeatin concentrations and time of incubation. Release of NO was inhibited by the NO scavenger, PTIO (2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxy-3-oxide), showing that the fluorescence formation was NO specific. An animal nitric oxide synthase inhibitor, AET (2-aminoethyl-2-thiopseudourea), inhibited NO formation in both genotypes, arguing for the presence of an animal NOS-like enzyme in plants. Using the cell-permeable NO-binding dye DAR-4M AM (diaminorhodamine-4M acetoxymethyl ester), NO-dependent fluorescence formation in the plants tissue was studied. The zeatin-dependent NO-induced fluorescence formation was observed in leaf blades, veins, trichomes, hydathodes, stomata, vascular bundles and root meristems. In the *nia1 nia2* seedlings, which lack one important plant NO producing enzyme NR, the tissue distribution of NO biosynthesis and the physiological response to cytokinin was changed in comparison to wild type plants. Differences in NO-induced fluorescence between the *nia1 nia2* and wild type plants were observed in the cotyledons, roots, and hypocotyls. Therefore, NR could not be the zeatin regulated NO producing enzyme. In order to investigate whether known cytokinin signaling proteins are necessary for cytokinin-induced NO biosynthesis two knockout *Arabidopsis* lines, a cytokinin receptor *ahk4* (*Arabidopsis* histidine kinase) and a triple phospho-transfer protein *ahp1 ahp2 ahp3* (*Arabidopsis* histidine containing phospho-transfer), were studied. To investigate the contribution of a second known NO synthesis enzyme in plants, *At*NOS1 (*Arabidopsis thaliana* nitric oxide synthase), to zeatin-induced NO biosynthesis a knockout line of this *Atnos1* gene was used. The knockout line showed an indistinguishable NO-dependent fluorescence formation from wild type plants, which argues against a role of this particular enzyme in the cytokinin action. As *AtNOS1* belongs to a small gene family of six homologous genes in *Arabidopsis* the other five genes and proteins remain to be investigated. The cytokinin receptor *ahk4* knockout produced less NO in the main root bundle where this receptor is reported to be expressed. A triple *ahp1 ahp2 ahp3* knockout line showed an impaired response to zeatin. No accumulation of NO in the roots, hypocotyls, and cotyledons of the triple knockout in response to zeatin were observed while NO accumulation in the first true leaves showed a stronger response to zeatin than that of wild type. The zeatin-untreated triple knockout seedlings showed no clear difference in

NO-dependent fluorescence formation in comparison to wild type. In conclusion, cytokinin signaling components are necessary for the zeatin-induced NO biosynthesis. When the effect of PAs on NO biosynthesis was investigated, spermine and spermidine increased NO release in the tobacco cell cultures and *Arabidopsis* seedlings were observed. Spm was observed as the most active PA and it also stimulated NO release with no apparent lag phase. These responses were quenched by addition of AET and PTIO. Spm and Spd increased NO biosynthesis in the elongation zone of the *Arabidopsis* root tip, where putrescine and arginine had a little or no effect. PAs could either act as signals to regulate NO biosynthesis or perhaps, as substrates to NO biosynthetic enzyme(s) in plants.

Key words: Nitric oxide, Cytokinin, Polyamines, *Arabidopsis*, signal transduction.

KURZFASSUNG

In der vorgelegten Arbeit wurde der Effekt von Zeatin und verschiedenen Polyaminen auf die Stickoxid (NO)-Biosynthese in *Arabidopsis thaliana*-Keimlingen und *Nicotiana tobacum*-Zellen untersucht. Durch den Einsatz des die Zellmembran nicht durchdringenden Farbstoffes DAR-4M (Diaminorhodamin-4M) wurde die Freisetzung von NO in das Inkubationsmedium durch Bindung an den Farbstoff und Fluoreszenz-Erhöhung gemessen. Eine von Zeatin stimulierte NO-Produktion wurde sowohl im *Arabidopsis*-Wildtyp als auch in einer Doppelmutante für die Nitratreduktase-Enzyme *nia1 nia2* beobachtet. Diese NO-Synthese war abhängig von der Zeit und Konzentration des Hormons. Die Freisetzung von NO wurde durch den spezifisch NO-bindenden Stoff PTIO (2-Phenyl-4,4,5,5-Tetramethylimidazolin-1-oxy-3-oxide) gehemmt. Dies zeigte, dass die Fluoreszenzbildung NO-spezifisch war. Der Stickoxid-Synthase (NOS) Hemmstoff AET (2-Aminoethyl-2-Thiopseudoharnstoff) blockierte die NO-Bildung in beiden Genotypen, was für die Präsenz von tierischen NOS-ähnlichen Enzymen in Pflanzen spricht. Durch den Einsatz des die Zellmembran durchdringenden, NO-bindenden Farbstoffes DAR-4M AM (Diaminorhodamine-4M-Acetoxymethylester) konnte die Zeatin-abhängige NO-induzierte Fluoreszenz in Blättern, Keimblättern, Blattnerven, Trichomen, Hydathoden, Stomata, Gefäßbündeln und in Wurzelmeristemen beobachtet werden. In *nia1 nia2* Keimlingen, denen das wichtige Enzym Nitratreductase (NR) fehlte, waren die Gewebeverteilung der NO-Biosynthese und die physiologische Reaktion auf Cytokinin im Vergleich zu Wildtyp-Pflanzen verändert. Unterschiede in NO-induzierter Fluoreszenz zwischen *nia1 nia2* und Wildtyp-Pflanzen wurden in den Keimblättern, Wurzeln und Hypokotylen gefunden. Um den Einfluss des Pflanzenenzyms *AtNOS1* (*Arabidopsis thaliana* nitric oxide synthase) auf die Zeatin-induzierte NO-Biosynthese zu erforschen, wurde eine Knockout-Linie für dieses Gen (AT3g 47450) benutzt. Die Knockout-Linie zeigte eine normale physiologische Reaktion auf Zeatin, die vom Wildtyp nicht zu unterscheiden war. Das spricht gegen die Rolle dieses Enzyms in der Cytokinin-induzierten NO-Biosynthese. *AtNOS1* gehört zu einer kleinen Gen-Familie von sechs homologen Genen in *Arabidopsis*; die anderen fünf Gene und Proteine müssen noch erforscht werden. Da *nia1 nia2* Mutanten innerhalb von Minuten auf Zeatin mit erhöhter NO-Synthese reagierte, konnte NR nicht das NO-produzierende Enzym sein, das von Zeatin reguliert wird. Um herauszufinden, ob die bekannten Proteine der Cytokinin-Signaltransduktion für eine Cytokinin-induzierte NO-Biosynthese notwendig sind, wurden zwei Knockout-*Arabidopsis*-Linien für diese Proteine untersucht. Die Knockout-Linie für den Cytokinin-Rezeptor, dreifache *ahk4* (*Arabidopsis* histidine kinase), produzierte in der Hauptwurzel weniger NO. Die dreifache Knockout-Linie *ahp1 ahp2 ahp3* (*Arabidopsis* histidine containing phospho-transfer protein) zeigte eine

geänderte Reaktion auf Zeatin. Eine Akkumulation von NO-induzierter Fluoreszenz nach Zeatin-Applikation blieb in den Wurzeln, Hypokotylen und Keimblättern aus, während die Primärblätter eine stärkere Reaktion zeigte als der Wildtyp. Nicht mit Zeatin behandelte Keimlinge zeigten keine klaren Unterschiede im Vergleich zum Wildtyp. Die Schlussfolgerung ist: Proteine der Cytokinin-Signaltransduktion sind notwendig für eine Zeatin-induzierte NO-Biosynthese. Bei der Untersuchung des Effekts von Polyaminen auf die NO-Biosynthese wurde beobachtet, dass die Spermin und Spermidin die Freisetzung von NO in den Tabak- Zellkulturen und in *Arabidopsis* Keimlingen deutlich erhöhten. Die Vorstufe in der Biosynthese der Polyamine, Arginin und Putrescin, zeigten nur geringe Effekte. Spermin war das aktivste; es stimulierte auch die Freisetzung von NO ohne eine sichtbare Verzögerung. Die Reaktion wurde durch Zugabe von AET und von PTIO gehemmt. Spermin und Spermidin erhöhten die NO-Biosynthese in der Elongationszone der Wurzelspitze von *Arabidopsis*, während Putrescin und Arginin keinen beziehungsweise nur einen kleinen Effekt zeigten. Polyamine könnten entweder als die NO-Biosynthese regulierende Signale wirken oder als Substrate von Enzymen der NO-Biosynthese in Pflanzen. Letzteres könnte als Indiz für ein neues Enzym der NO-Biosynthese der Pflanzen sein.

Stichwörter: Stickoxid, Cytokinin, Polyamine, *Arabidopsis*, Signaltransduktion.

ABBREVIATIONS

AET	**2-aminoethyl-2-thiopseudorea**
AHK	***Arabidopsis* histidine kinase**
ahk4	***Arabidopsis* histidine kinase 4 knockout**
AHP	***Arabidopsis* histidine containing phospho-transfer protein**
ahp1 ahp2 ahp3	***Arabidopsis* histidine containing phospho-transfer protein triple knockout**
Arg	**arginine**
ARR	***Arabidopsis* response regulator**
***At*NOS**	***Arabidopsis* nitric oxide synthase**
Atnos	***Arabidopsis* nitric oxide synthase knockout**
BY2	**bright yellow 2**
cGMP	**3´,5´-cyclic guanosine monophosphate**
CKX	**Cytokinin oxidase/dehydrogenase**
DAR-4M AM	**diaminorhodamine-4M acetoxymethyl ester**
DAR-4M	**Diaminorhodamine-4M**
H_2O_2	**hydrogen peroxide**
HP	**histidine containing phospho-transfer protein**
h	**hour**
HR	**hypersensitive response**
IPT	**isopentenyl transferase**
KPO_4	**potassium phosphate**
MES	**2-Morpholinoethanesulfonic acid, monohydrate**
min	**minute**
MS-Medium	**Murashige and Skoog medium**
nia1 nia2	**nitrate reductase double mutant**
NO	**nitric oxide**

NO_2	**nitrogen dioxide**
NOS	**nitric oxide synthase**
NR	**nitrate reductase**
$O_2^{\cdot -}$	**superoxide anion**
PAs	**polyamines**
PTIO	**2-phenyl-4, 4, 5, 5-tetramethylimidazoline-1-oxy-3-oxide**
Put	**putrescine**
ROS	**reactive oxygen species**
SNAP	**N-(ß-D-glucopyranosyl)-N^2-acetyl-S-nitroso-D, L-penicillaminamide**
Spd	**spermidine**
Spm	**spermine**
WT	**wild type**
DAF-2	**4,5-diaminofluorescein**

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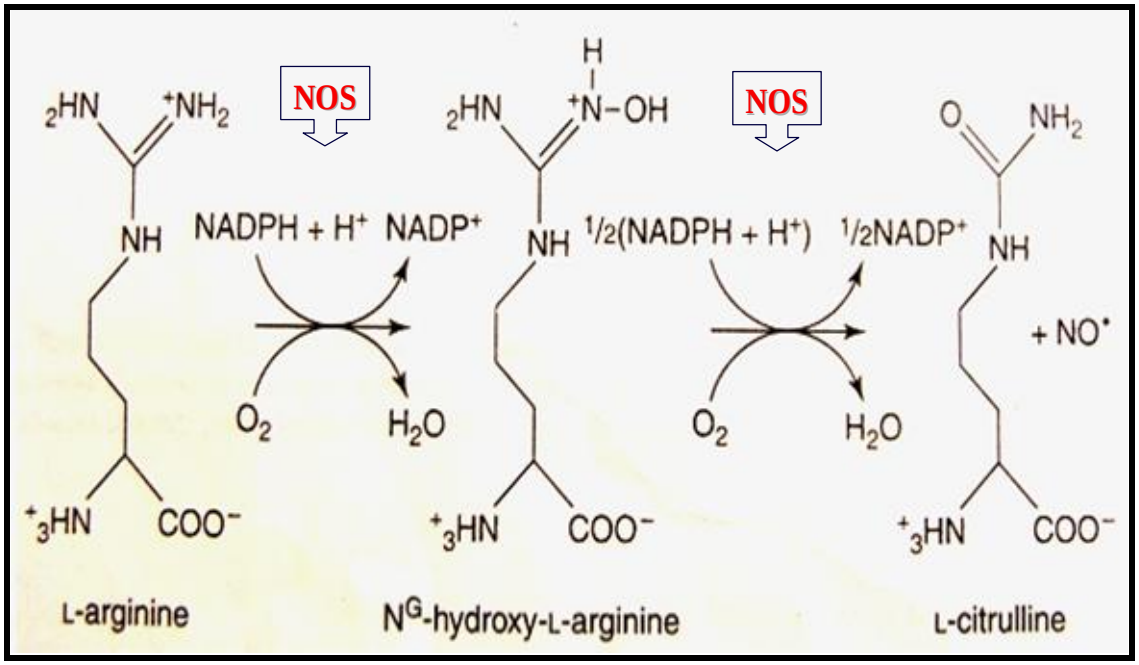
1 INTRODUCTION

1.1 NO-Biosynthesis in Plants and Animals

Nitric oxide is a small, highly diffusible gas and a ubiquitous bioactive molecule which is involved in the regulation of diverse physiological and pathophysiological mechanisms in animals and plants (Stamler et al., 1992; Wojtaszek, 2000; Neill et al., 2003; Lamattina et al., 2003; Crawford and Guo, 2005). In 1992, NO was named "Molecule of the Year" by *Science* because of its widespread biological significance, and since then there has been a huge number of studies on NO biology (Koshland, 1992). In recent years NO has emerged as a new chemical messenger in plant biology. NO is involved in hormone signaling (abscisic acid, auxin, and cytokinin), light responses, flowering, pollen tube orientation, gravitropism, antisenescence and plant defense responses (Leshem and Haramarty, 1996; Lamattina et al., 2003; Neill et al., 2003; He et al., 2004; Wendehenne et al., 2004; Prado et al., 2004; Crawford and Guo, 2005; Hu et al., 2005).

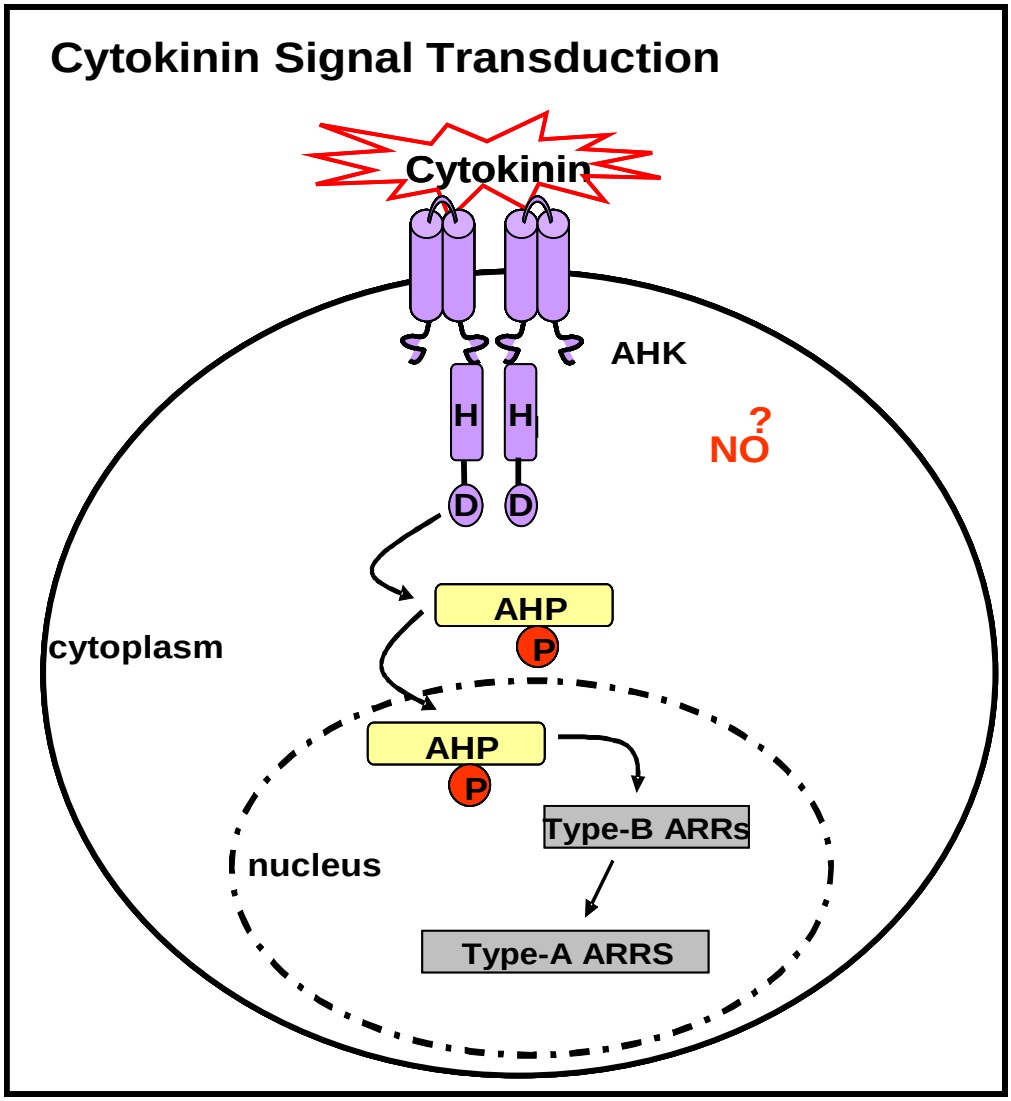
NO is synthesised by nitric oxide synthase (NOS) from L-arginine, O_2 and NADPH (Scheme 1) in animals. Arg is first converted to hydroxyarginine, a nonreleased catalytic intermediate, with the final products being citrulline and nitric oxide (Alderton et al., 2001). Although NO synthesis is well characterized in animals (Alderton et al., 2001; Crawford and Guo, 2005), NO biosynthesis is not well understood in plant yet. Several potential sources of NO in plants have been reported, NOS (Guo et al., 2003) and NR (Harper, 1981; Dean and Harper, 1988; Yamasaki, 2000; Garcia-Mata and Lamattina, 2003), nitrite: NO-reductase (Stöhr et al., 2001), xanthine oxidoreductase (Harrison, 2002), and non-enzymatic sources (Conney et al., 1994; Bethke et al., 2004). NR can produce NO and N_2O from nitrite resulting in NO emission (Harper, 1981; Dean and Harper, 1986; Yamasaki and Sakihama, 2000; Rockel et al., 2002). *Arabidopsis* NOS (*At*NOS) was reported to use arginine as substrate. *AtNOS1* belongs to a small gene family of six genes in the *Arabidopsis* genome which are related to the NOS in the snail *Helix pomatia* (Guo et al., 2003). However, in plants, no gene or protein whose sequence is similar to the higher animal NOS protein has been found in the sequenced *Arabidopsis* genome. The biology of NO biosynthesis in plants seems to be quite different from that of animals.



Scheme 1. Biosynthesis of nitric oxide in animals. The nitric oxide radical is generated through five-electron oxidation of L-arginine via the N^G-hydroxy-L-arginine intermediate in the presence of NADPH and oxygen. The reaction is catalysed by nitric oxide synthase (NOS). The other reaction products are citrulline and NADP (Alderton et al., 2001).

1.2 Cytokinin signaling and the role of NO

Cytokinins are N^6-substituted adenine derivatives that play a role in almost all aspects of plants growth and development, including cell division, vascular development, chloroplast differentiation and senescence (Mok and Mok, 2001). Cytokinin perception and signaling (Scheme 2) are reported to be similar to the bacteria two-component phosphorelays. Activation of the cytokinin receptors *Arabidopsis* histidine kinase (AHK) by cytokinin binding is postulated to result in the phosphorylation of the *Arabidopsis* histidine containing phospho-transfer (AHP) proteins, which move from the cytoplasm into the nucleus, where they transfer a phosphate moiety to *Arabidopsis* response regulators (ARRs), and leading to the cellular responses (Inoue et al., 2001; Hwang and Sheen, 2001; Hutchison and Kieber, 2002).



Scheme 2. The cytokinin signal transduction. AHK (histidine protein kinases) serves as a cytokinin receptor and AHPs (histidine phosphotransfer proteins) transmit the signal from AHK to the nuclear response regulators (ARRs), which can activate or repress transcription (Inoue et al., 2001; Hwang and Sheen, 2001; Hutchison and Kieber, 2002). In which step NO is involved in this pathway is still an open question.

Although there is an ever-increasing number of NO responses in plants, relatively little knowledge on the relation between NO and plant hormone signal transduction is gathered. It has been reported that cytokinin induced NO biosynthesis in tobacco BY-2 and *Arabidopsis* cells (Tun et al., 2001; Carimi et al., 2005) and NO may have a role in cytokinin-mediated betalaine accumulation in *Amaranthus* (Scherer and Holk, 2000). Scherer (2004) has proposed a role of NO as a potential second messenger in cytokinin signaling. The first part of this study focuses on the role of cytokinin signaling components (AHK4 and AHPs) and nitric oxide biosynthesis (NR and

*At*NOS) enzymes in cytokinin-induced NO production.

1.3 Polyamines and NO Biosynthesis

PAs are naturally occurring low molecular weight, poly-cationic, aliphatic nitrogenous compounds present in all living cells. As PAs are basic molecules, which are positively charged at physiological pH, they can bind strongly to negatively charged macromolecules like nucleic acids, acidic phospholipids and many types of proteins including numerous enzymes whose activities are directly modulated by PAs binding. These ionic interactions are important in regulating the structure and function of biological macromolecules, as well as their synthesis (Bouchereau et al., 1999). The most commonly found PAs in plant cells are the tri-amine spermidine (Spd), the tetra-amine spermine (Spm) and their precursor di-amine putrescine (Put). They are usually present in the plant cell at significantly higher levels than plant hormones and their endogenous concentrations required for biological effects are in millimolar range (Bouchereau et al., 1999; Sebela et al., 2001). In general, prokaryotic cells contain fairly large amounts of Put, small quantities of Spd and no Spm, while eukaryotes have little Put, more Spd and considerable amount of Spm. There is evidence that PAs are taken up by cell suspension culture (Bais and Ravishankar, 2002). In spite of their higher concentration than those of phytohormones, PAs are reported to mediate the action of hormones as part of their signal response and thus are regarded as hormonal mediators (Flores and Filner, 1985; Slocum and Flores, 1991; Altman and Levin, 1993).

PAs are involved in various aspects of cellular responses such as cell division, root formation, development of flowers and fruits, zygotic and somatic embryogenesis, retardation of senescence, dormancy breaking of tubers and germination of seeds (Feirer et al., 1984; Evans and Malmberg, 1989; Galston and Sawhney, 1990; Galston et al., 1995; Kumar et al., 1997; Walden et al., 1997; Bouchereau et al., 1999; Kakkar et al., 2000; Bais and Ravishankar, 2002). PAs have been suggested to afford protection against a large variety of environmental stresses, including salinity, chilling, ozone exposure and potassium deficiency (Bouchereau et al., 1999). The protective function of PAs is mainly due to their cationic nature at cellular pH. By

binding to proteins and lipids, PAs can stabilize cellular structures such as thylakoid membranes (Tiburcio et al., 1994). PAs have also been proposed to act as radical scavengers (Bouchereau et al., 1999) and as regulators of potassium channels in stomata (Liu et al., 2000). More recently, it was observed that Spm plays a role as a mediator in defence signaling against pathogens (Takahashi et al., 2003; Takahashi et al., 2004), accumulates during defence (Mo and Pua, 2002) and enhances resistance (Yamakawa et al., 1998).

Oxidation of PAs is important for the metabolism and regulation of proper functioning of PAs. Regulation of the level of interacellular PAs involves degradation of Put by copper-containing diamine oxidases and of Spd and Spm by flavin adenine dinucleotide-containing polyamine oxidases. These enzymes predominantly occur in the apoplast and may also function in peroxidative lignification of the cell wall, the cell wall stiffening, and the cellular defence (Sebela et al., 2001; Agostinelli et al., 2004). Di-amine oxidases are involved in the oxidative deamination of diamines like Put or cadaverine on their primary amino groups and produce aminoaldehyde, ammonia and hydrogen peroxide. On the other hand, polyamine oxidases are thus the key enzymes for the catabolism of PAs which catalyse the oxidation of the secondary amino group of Spm, Spd and their acetyl derivatives results in the production of the corresponding aldehyde, 1,3-diaminopropane and hydrogen peroxide (Maccarrone et al., 2001).

Although animal nitric oxide synthase (NOS) which uses Arg as a substrate in animal systems has been well characterized, the enzymatic NO production in plant seems to be more complex and is still not clearly understood yet. However, in plants the relation between PA and NO biosynthesis has not been studied yet. NO biosynthesis by cytokinin in plant cell cultures of *Arabidopsis*, parsley and tobacco (Tun et al., 2001; Scherer, 2004; Carimi et al., 2005) and NO mimicked cytokinin-induced betalaine accumulation in *Amaranthus caudatus* seedlings (Scherer and Holk, 2000) have been reported. Since the biology of PAs and cytokinin in plants seems to possess many overlapping physiological functions (Greenland and Lewis, 1984; Galston et al., 1995; Laxalt et al., 1997; Cohen, 1998), this initiated the investigation of the potential relationship between PAs and NO in plants. Therefore, in the second part of this study,

the regulation of NO biosynthesis by different PAs was investigated and their NO production was compared to NO biosynthesis by Arg, which is a substrate for *At*NOS. The aim of present work is to investigate the regulation of NO biosynthesis by different PAs.

2 MATERIALS AND METHODS

2.1 Plant Materials and Growing Conditions

Columbia ecotype *Arabidopsis thaliana* wild type, *nia1 nia2* double mutant (Wilkinson and Crawford, 1993), *ahk4* knockout (gift from Dr. J. Kieber), *ahp1 ahp2 ahp3* triple knockout (phosphotransfer protein) (gift from Dr. J. Kieber) and *Atnos1* (gift from Dr. N. Crawford) knockout seedlings were used in the experiments. *Arabidopsis* seeds were surface-sterilized (Appendix ii) and maintained for 3 days at +4 $^{\circ}$C for vernalization. Twenty *Arabidopsis* seeds per well were grown in half-strength liquid MS (Murashige and Skoog, 1962) medium (appendix i). The growth conditions were a 16-h photoperiod, photon flux of 20-23 μmol m^{-2} s^{-1}, and 22.5 $^{\circ}$C on a rotatory shaker (60 rpm). Seven to eight days old seedlings were used in the experiments. Tobacco strain BY-2 was grown for 7 days in MS medium in the dark at 26 $^{\circ}$C on a rotatory shaker (125 rpm) (Tun et al., 2001).

2.2 Fluorometric Assay for NO-dependent Relative Fluorescence Units Measurement

NO biosynthesis was measured using a modification of the previously described method (Tun et al., 2001). The cell-impermeable dye DAR-4M (diaminorhodamine-4M) (Kojima et al., 2000 and 2001) was used. This dye reacts with NO in a pH-independent manner at pH>4. Therefore, DAR-4M can be used in the slightly acidic (pH 5.7) plant growth media.

For the assay of the zeatin-induced NO production, twenty *Arabidopsis* seedlings were incubated with DAR-4M (1 μM or 2.5 μM) including different concentrations of zeatin (Appendix. iii) in 125 μM KPO_4 buffered at pH 5.7 (Appendix iv). The incubation condition of the seedlings was in the light at 22.5 $^{\circ}$C on a rotatory shaker (60 rpm) in this medium.

For the measurement of the PA-induced NO production, tobacco cells were weighed into 100 mg lots of fresh weight per experiment. Samples were transferred into 2 ml of 30 mM MES pH 5.7 (Appendix v) containing PAs (Put, Spd, Spm) or Arg (Appendix

iii) and different concentrations of the inhibitor AET plus 2.5 µM DAR-4M. *Arabidopsis* seedlings were incubated in the light, on a rotatory shaker (60 rpm), and tobacco cells were incubated in the dark (125 rpm). At 0 h, 1 h, 2 h, 3 h and 4 h incubation time, 200 µl of the supernatant was taken and diluted to 2 ml with 1800 µl of double distilled water. To check the chemical effect of PAs and H_2O_2 on dye fluorescence formation, the PAs were dissolved in 30 mM MES pH 5.7 and incubated without plants in the presence of 2.5 µM DAR-4M for 2 h at 60 rpm.

The supernatant was taken at each time point and the relative fluorescence was measured with an excitation at 560 nm and emission at 575 nm in a LS-5 luminescence spectrometer (Perkin-Elmer, Überlingen, Germany). The value of each 0 time point measurement was subtracted. All experiments were repeated 2-5 times with similar results and the data points represent one to three replications.

2.3 Characterization of NO Formation in *Arabidopsis* Seedlings

The tissue pattern of NO production in the plant was investigated by binding of NO to the cell-permeable derivative DAR-4M AM and then recorded under a fluorescence microscope. For the experiment of two-day treatments with cytokinin, seven-day old wild type *Arabidposis* seedlings (20 seedlings for each treatment) were grown in liquid MS medium. The seedlings were loaded with 1 µM dye for 4 h, washed twice with double-distilled water and then incubated with zeatin (0 µM or 5 µM) and PTIO (0 mM or 1 mM) for 48 h in 125 µM KPO_4, buffered at pH 5.7. The incubation condition was under the light at 22.5 $^{\circ}$C on a rotatory shaker (60 rpm). To end the incubation, the seedlings were washed twice with double-distilled water again and kept at +4 $^{\circ}$C prior to the microscopy. For experiments with a two-week treatment with zeatin, seedlings were grown for 10 to 14 days on solid MS medium (1.2 % agar), containing different concentrations of zeatin and with or without the cell-permeable NO specific fluorescent dye DAR-4M AM (2.5 µM or 5 µM). Seedlings were harvested 10-14 days after germination for microscopy. The leaves, cotyledons, hypocotyls were separated gently and slides were prepared.

To study the tissue pattern of PA-induced NO formation, seedlings were loaded with 1 µM DAR-4M AM for 4 h in 30 mM MES buffer, pH 5.7. The dye solution was taken

away and then seedlings were washed twice with double-distilled water. Then the seedlings were incubated with PAs or Arg (1 mM) in 30 mM MES buffer at pH 5.7 for 18 h. Incubation was in the light at 22.5 °C on a rotatory shaker (60 rpm). Then seedlings were washed again twice with water prior to observation. Samples were prepared on microscopic slides.

The fluorescence formation in the plants was recorded with an Axioskop2 Mot microscope coupled to an AxioCam and a program AxioVision Rel. 4. 2 using a filter set 20. The excitation was set at BP 546/12 nm; the beam splitter at FT 560 nm; the emission at BP 575-640 nm (Carl Zeiss, Oberkochem, Germany). The digital images to be compared were recorded exactly at the same camera settings and were not further processed for each comparison.

2.4 Analysis of Morphological Responses of *Arabidopsis seedlings* to Zeatin

In order to compare the zeatin concentration dependence of the phenotypic responses of all genotypes, seedlings were grown for 10 to 14 days on solid MS medium, containing different concentrations of zeatin. *Arabidopsis* seedlings, 10 to 14 days old, grown on solid MS medium were harvested. Leaves, cotyledons, hypocotyls and roots were separated gently and scanned by an Epson perfection 1250 scanner with a program Epson smart panel at 800-1200 dpi resolution. The lengths or areas of scanned plant segments were measured using an Axiovision Rel 4.2 program (Carl Zeiss, Oberkochem, Germany).

3 RESULTS

3.1 Methodological Tests

3.1.1 Seed Purity Test

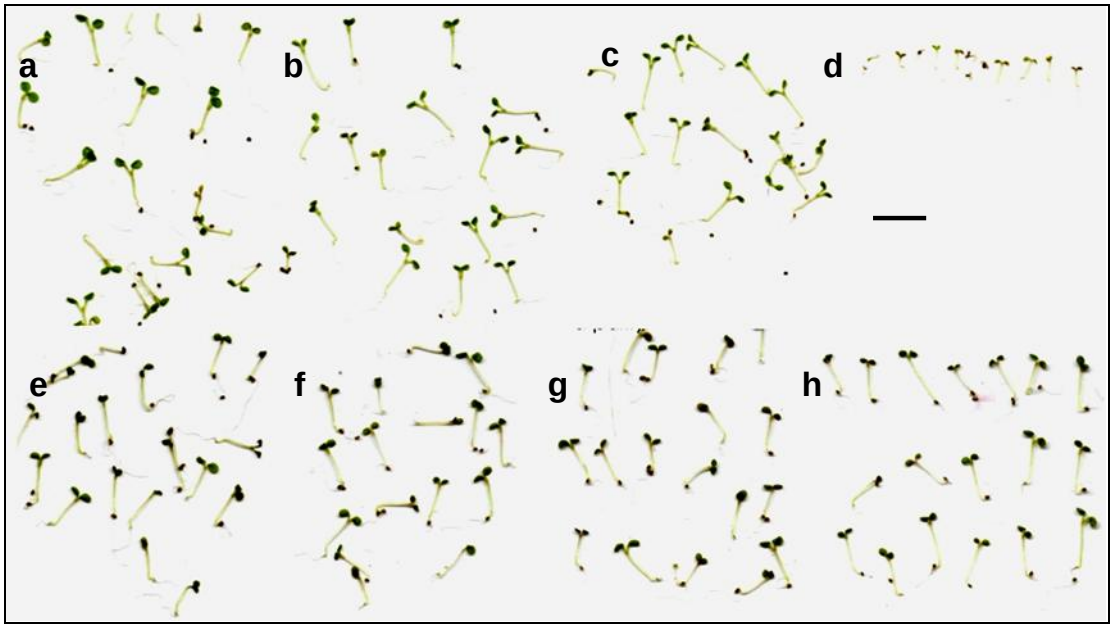


Fig. 1. The purity test of the *nia1 nia2* double mutant *Arabidopsis* seedlings. Wild type and mutant *Arabidopsis* seedlings were grown for 6 days in half strength liquid MS medium containing different concentrations of chlorate ($KClO_3$). The growth condition of seedlings was documented by scanning. (a-d): wild type seedlings; (e-h): double mutant; (a and e): no chlorate; (b and f): 0.5 mM $KClO_3$; (c and g): 1 mM $KClO_3$ and (d and h): 5 mM $KClO_3$. Bar = 10 mm.

The *nia1 nia2* mutant was new to our laboratory. Therefore, the seed purity was tested. Nitrate is an important nutrient for plants. The nitrate reductase, a cytosolic enzyme required for nitrate utilization, reduces nitrate to nitrite (NO_3^- à NO_2^-). Nitrite then is further reduced to ammonium (NH_4^+) by the chloroplast enzyme nitrite reductase (Taiz and Zeiger, 1998). Chlorate is the chlorine analog of nitrate, and it is taken up and then reduced to toxic chlorite by nitrate reductase. The nitrate reductase double mutant plant *nia1 nia2* is defective in the assimilation of nitrate. Resistance to the herbicide chlorate (Wilkinson and Crawford, 1993) can be used to select nitrate reductase mutants. So, mutant plants did not reduce chlorate to toxic chlorite and survived at high concentration of chlorate level while wild type plants were severely

damaged (Fig. 1 a-h). Therefore, the seed purity of *nia1 nia2* mutant was confirmed here.

3.1.2 Comparison Between Relative Fluorescence Units Based on the Seedling Number, the Fresh Weight and the Protein Content

In order to set up the most suitable method for the measurement of NO released from *Arabidopsis* seedlings, the fluorescence units based on (1) seedling number, (2) the fresh weight, and (3) the protein content were compared. Twenty wild type *Arabidopsis* seedlings (equivalent to 88.9 $\pm$ 7.7 mg fresh weight) were used for each sample (Fig. 2). The relative fluorescence units of NO released per sample were measured. Then the fresh weight and protein content of the sample was recorded. The fluorescence units of NO released were calculated in three different ways. When the average of units of 12 identical lots of 20 seedlings each was set as 100%, a standard deviation of $\pm$ 10.85% was obtained. When the units (with the same samples) based on fresh weight were calculated, the average was 100% $\pm$ 13.41%. The protein content of the same sample was extracted with 0.5 M potassium hydroxide (KOH) and measured by an assay according to Bradford (1976). When the units were based on protein extracted, 100% $\pm$ 19.45% was obtained. Therefore it could be concluded here that relative fluorescence units based on seedling number had the lowest variance of data within an experiment.

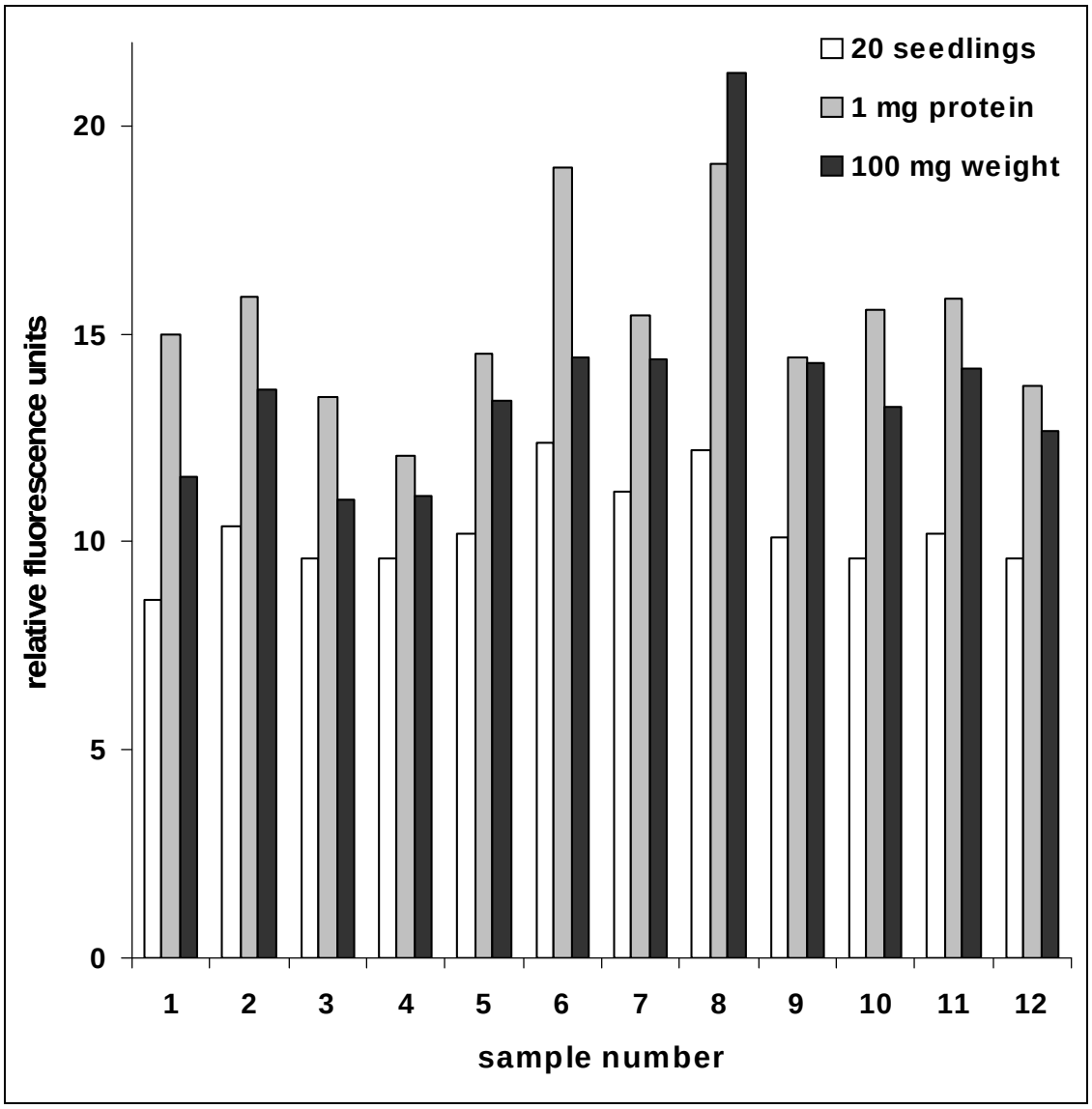


Fig. 2. Comparison between the NO-dependent relative fluorescence units based on the seedling number, the fresh weight and the protein content of the 12 identical samples. The relative fluorescence due to NO formation was measured in the presence of the 1 µM NO specific dye DAR-4M. Twenty wild type *Arabidopsis* seedlings for each sample were used. The relative fluorescence was measured at an excitation of 560 nm and an emission of 575 nm. Data are expressed as fluorescence units per 20 seedlings (white bars), units per mg protein (grey bars), and units per 100 mg fresh weight (black bars).

3.1.3 Comparison Between the Filter Sets 14 and 20 According to their Abilities to Cut off Autofluorescence from Chlorophyll

The filter set 14 (Fig. 3 a) failed to differentiate between the autofluorescence and the NO-dependent fluorescence formation of DAR-4M AM (Fig. 3 c). The bright red autofluorescence from chlorophyll was also observed at the higher magnification when photos were taken with an excitation wavelength of 510-560 nm together with 590 nm emission filter set 14 (Fig. 3 c). Therefore the filter set 20 with an excitation wavelength of 546/12 nm together with 575-640 nm emission (Fig. 3 b) was introduced here and its ability to cut off autofluorescence from chlorophyll was checked by comparing these two filter sets (Fig. 3 c and d). The filter set 20 reduced or eliminated the red autofluorescence from chlorophyll and permitted to observe the clear NO-dependent fluorescence formation by DAR-4M AM (Fig. 3 d). The dye-treated leaf was showing the fluorescence formation in veins and trichomes while without dye the leaves were rather dark. So it was concluded here that the filter set 20 could effectively cut off the autofluorescence from chlorophyll in the Zeiss microscope.

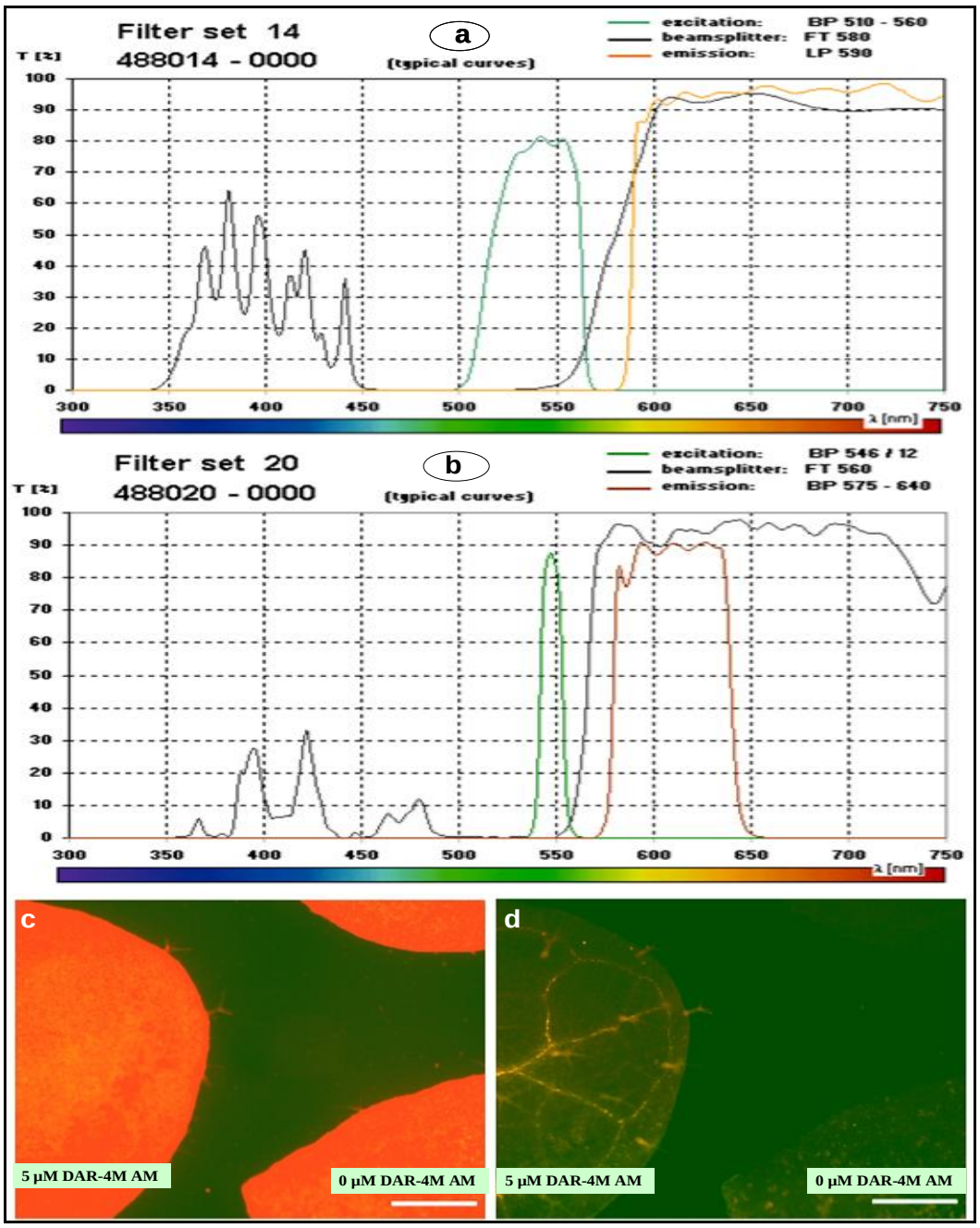


Fig. 3. Comparison between the filter sets 14 and 20 of their abilities to cut off the autofluorescence from chlorophyll. The curves for the excitation and the emission wavelengths of (a) the filter set 14 with an excitation wavelength of 510-560 nm together with 590 nm emission and (b) the filter set 20 with an excitation wavelength of 546/12 nm together with 575- 640 nm emission filter set 20. (a) and (b) were received from http://www.zeiss.de/ (Sept. 2005). The leaves of two weeks old *Arabidopsis* seedlings grown on the solid MS medium containing (0 µM or 5 µM) DAR-4M AM. The images (c) and (d) were taken from identical leaves. The single leaf from the left side was grown with 5 µM DAR-4M AM and two leaves from the right side were grown with 0 µM DAR-4M AM. (c) The images of the leaves were taken with the filter no 14 and (d) the images of the leaves were taken with the filter set 20. Bar = 500 µm.

3.2 Zeatin and NO Production in *Arabidopsis* Seedlings

3.2.1 NO Biosynthesis in *Arabiodpsis* Seedlings

3.2.1.1 Comparison of NO Release from Wild Type and *nia1 nia2* Double Mutant *Arabidopsis* Seedlings

Rapid regulation of NO biosynthesis is a prerequisite for its function as a second messenger in animal signal transduction (Mayer and Hemmes, 1997; Stamler et al., 2001). Rapid regulation of NO biosynthesis was also shown in several instances in plants or plant cells (Foissner et al., 2000; Tun et al., 2001; Lamotte et al., 2004; Zeidler et al., 2004). Here, rapid release of NO from cytokinin-treated wild type and *nia1 nia2* mutant *Arabidopsis* seedlings was measured to show that NO could act as a signal molecule in cytokinin action.

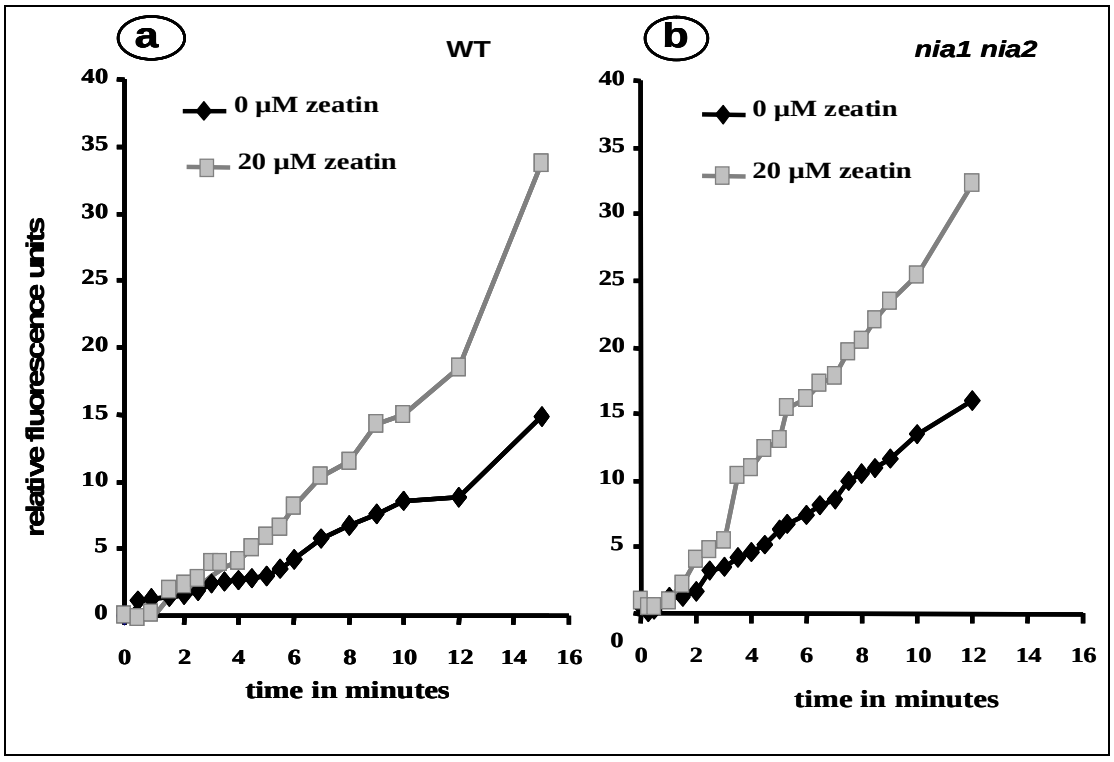


Fig. 4. Time kinetics of NO release in (a) wild type and (b) the *nia1 nia2 Arabidopsis* seedlings. The relative fluorescence of NO formation was measured by using 2.5 µM of the NO specific dye DAR-4M. Seven-day old *Arabidopsis* seedlings (20 seedlings for each treatment) and 0 µM or 20 µM zeatin were used. The relative fluorescence was measured at an excitation wavelength of 560 nm and an emission wavelength of 575 nm.

Release of nitric oxide from both wild type (Fig. 4 a) and *nia1 nia2* seedlings (Fig. 4 b) to the incubation medium was measured in this assay with the cell impermeable fluorescence dye DAR-4M. Both genotypes of *Arabidopsis* seedlings exhibited endogenous NO release which was rapidly enhanced by 20 µM zeatin after a 2-3 min lag phase. Cytokinin-induced NO-formation kinetics in both genotypes were similar, suggesting that NR might not be the enzyme responsible for the zeatin-regulated NO production.

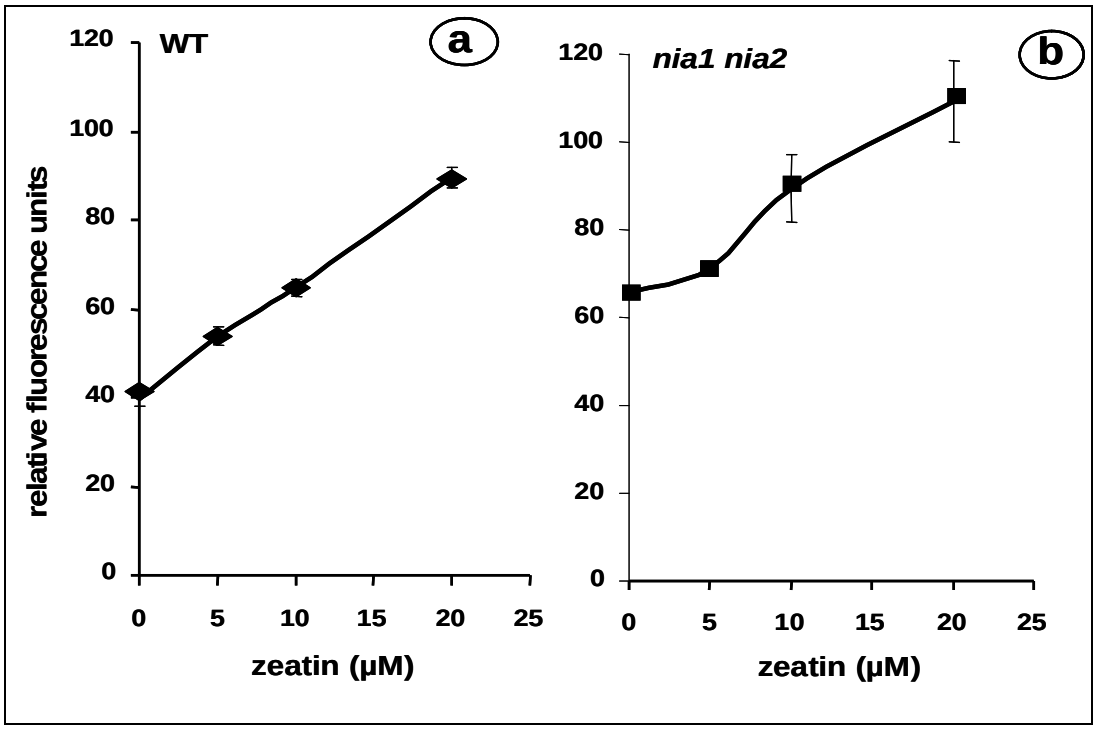


Fig. 5. Cytokinin concentration dependence of NO biosynthesis form (a) wild type and (b) the *nia1 nia2 Arabidopsis* seedlings. The relative fluorescence of NO formation was measured using the NO specific dye DAR-4M of a final concentration of 2.5 µM. Seven-day old *Arabidopsis* seedlings (20 seedlings for each treatment) were incubated with different concentrations of zeatin for 2 h. The relative fluorescence was measured at an excitation 560 nm and an emission 575 nm. (n=3; SEM).

NO release was dependent on the concentration of zeatin in both wild type (Fig. 5 a) and the *nia1 nia2* seedlings (Fig. 5 b). This cytokinin dependence NO production in both genotypes showed a similar pattern so that the response to zeatin of the NO production was not influenced by the absence of NR. The endogenous NO production was consistently higher in *nia1 nia2* seedlings.

3.2.1.2 NOS Inhibitor (AET) Inhibits NO Production and NO Scavenger (PTIO) Decreases NO-dependent Fluorescence Formation in Wild Type and *nia1 nia2 Arabidopsis* Seedlings

AET inhibited cytokinin-induced NO production in plant cells (Tun et al., 2001; Carimi et al., 2005). Here, both the endogenous and the cytokinin-induced nitric oxide production was inhibited by the animal nitric oxide synthase inhibitor AET, arguing for the presence of animal NOS-like enzymes in plants (Fig. 6 a and b). AET, which is an arginine analogue, could not be expected to inhibit activity of NR. The inhibition by the scavenger PTIO, specific for NO (Barchowsky et al., 1999), on both the endogenous and the cytokinin-induced fluorescence formation proved that the detected fluorescence formation was NO-specific (Fig. 6 c and d).

A similar inhibition by AET and PTIO of the NO release from wild type (Fig. 6 a and c) and the *nia1 nia2* (Fig. 6 b and d) mutant seedlings was observed. As NO release was inhibited by AET in the *nia1 nia2* mutant (Fig. 6 b), which is deficient in NR, it is more likely that the observed effects on cytokinin-induced NO formation were due to the presence of a NOS-like enzyme in the plants. PTIO is designed only as NO scavenger and cannot inhibit the activities of NO-producing enzymes. Here, PTIO inhibited both the endogenous and the cytokinin-induced NO production in the wild type and the *nia1 nia2 Arabidopsis* seedlings. These results indicate other enzymatic sources to produce NO in plants besides NR.

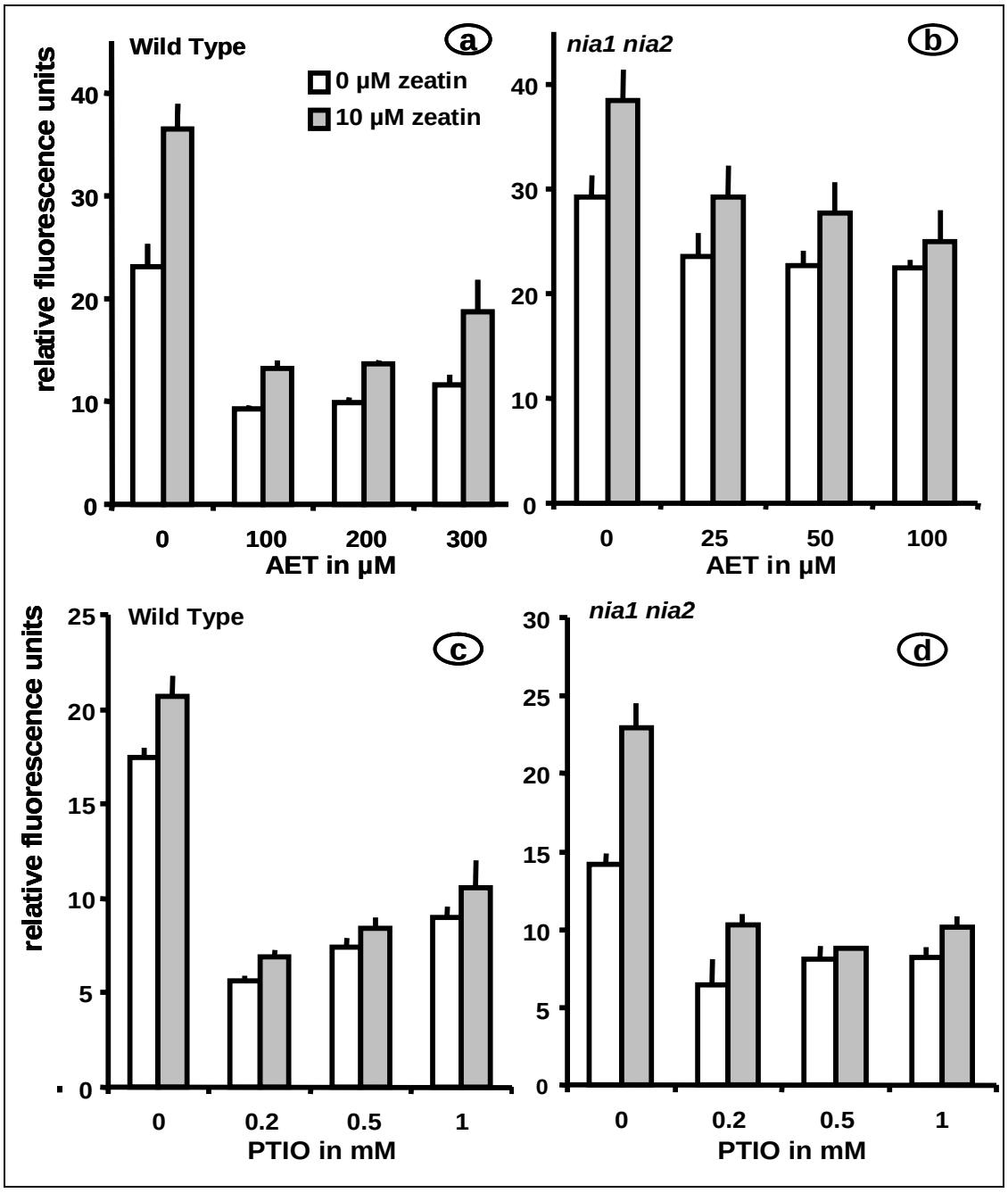


Fig. 6. Inhibition of AET and PTIO on NO released from (a and c) wild type and (b and d) the *nia1 nia2 Arabidopsis* seedlings. The relative fluorescence of NO formation was measured using the NO specific dye DAR-4M (2.5 µM). Seven-day old *Arabidopsis* seedlings (20 seedlings for each treatment) were incubated with or without 10 µM zeatin plus different concentrations of AET or PTIO for 2 h. The relative fluorescence was measured at an excitation 560 nm and an emission 575 nm.

3.2.1.3 Comparison of the Endogenous NO Released from Wild Type and *nia1 nia2* Double Mutant *Arabidopsis* Seedlings

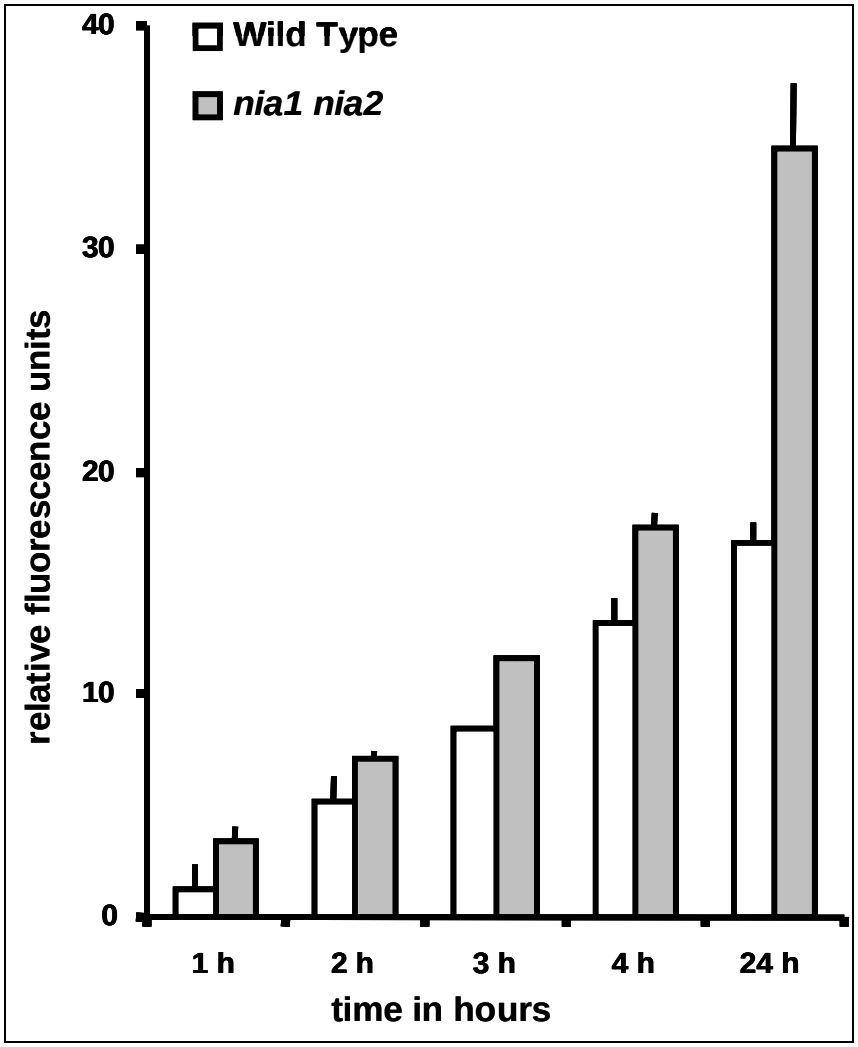


Fig. 7. Comparison of the time dependent endogenous NO released from the wild type and the *nia1 nia2* seedlings. The relative fluorescence of endogenous NO productions of 7-day old *Arabidopsis* seedlings (20 seedlings for each treatment) were measured by using the 2.5 µM DAR-4M. The relative fluorescence formation at each time point was recorded. The relative fluorescence units were measured using an excitation 560 nm and an emission 575 nm.

The endogenous NO production also during a longer incubation time in the fluorometric assay was studied in both genotypes. The measurement of NO production for up to two hours showed similar amounts in both genotypes (Fig. 7). Although the amount of endogenous NO released from wild type and the *nia1 nia2* seedlings during

the first two hours was not considerably different (Fig. 7), after 3 h incubation, the endogenous NO production in the *nia1 nia2* seedlings measurements was more clearly increased versus wild type. After 24 h incubation, the endogenous NO production in the *nia1 nia2* mutant was 158% $\pm$ 27.13% if that of value of the wild type seedlings was set to 100% (n = 6). Therefore, the endogenous NO production was observed to be considerably higher in the *nia1 nia2* mutant plants than that of wild type seedlings at 24 h measurement. A higher level of endogenous NO production in the *nia1 nia2* seedlings (Fig. 13-16) when grown for 14 days in dye containing agar was observed.

3.2.2 Fluorescence Imaging of Patterns of NO Formation in *Arabidopsis*

3.2.2.1 Cytokinin–induced Patterns of NO Production in Wild Type and *nia1 nia2 Arabidopsis* Seedlings

Although the NO production induced by zeatin could be observed by measuring the NO released from the whole plant into the incubation medium, this fluorometric assay could not visualize NO biosynthesis in different plant organs. In order to investigate the tissue-dependent NO biosynthesis pattern inside the plant, the cell-permeable NO fluorescent dye DAR-4M AM was employed. The wild type and the *nia1 nia2* double mutant *Arabidopsis* seedlings were grown for 2 weeks on solid MS medium containing 5 µM DAR-4M AM and the different concentrations of zeatin. The NO formation in plants was analysed in a Leica stereomicroscope with an excitation wavelength of 510-560 nm together with a 590 nm emission filter set 14 (Fig. 3 a).

In order to compare all four experimental conditions directly in a single picture, plants were cut into three parts, leaves, cotyledons, and hypocotyls plus root. In the leaves of both wild type and the *nia1 nia2* mutant seedlings, the NO dependent fluorescence was increased by addition of 1 µM and 5 µM zeatin (Fig. 8 c and d).With the same hormone additions, very little fluorescence formation was detected in leaves without dye proving that no disturbance of the results by autofluorescence of the chlorophyll (Fig. 8). This chloroplast-derived autofluorescence was strongly observed at higher magnification (Fig. 3 c). The patterns of NO formation in the leaves of both genotypes were similar. Cotyledons of wild type showed a weaker fluorescence formation than that of the *nia1 nia2* seedlings. No clear effect of increasing concentration of zeatin

was observed in cotyledons of both genotypes (Fig. 8 g and h). The size of cotyledons was bigger in response to zeatin which is a growth response to cytokinin in accordance with the data by Chory and coworkers (1994). In hypocotyls of both genotypes, NO formation was enhanced by addition of zeatin (Fig. 8 k and l). No further increase in NO production by higher cytokinin concentration was observed in these plant parts.

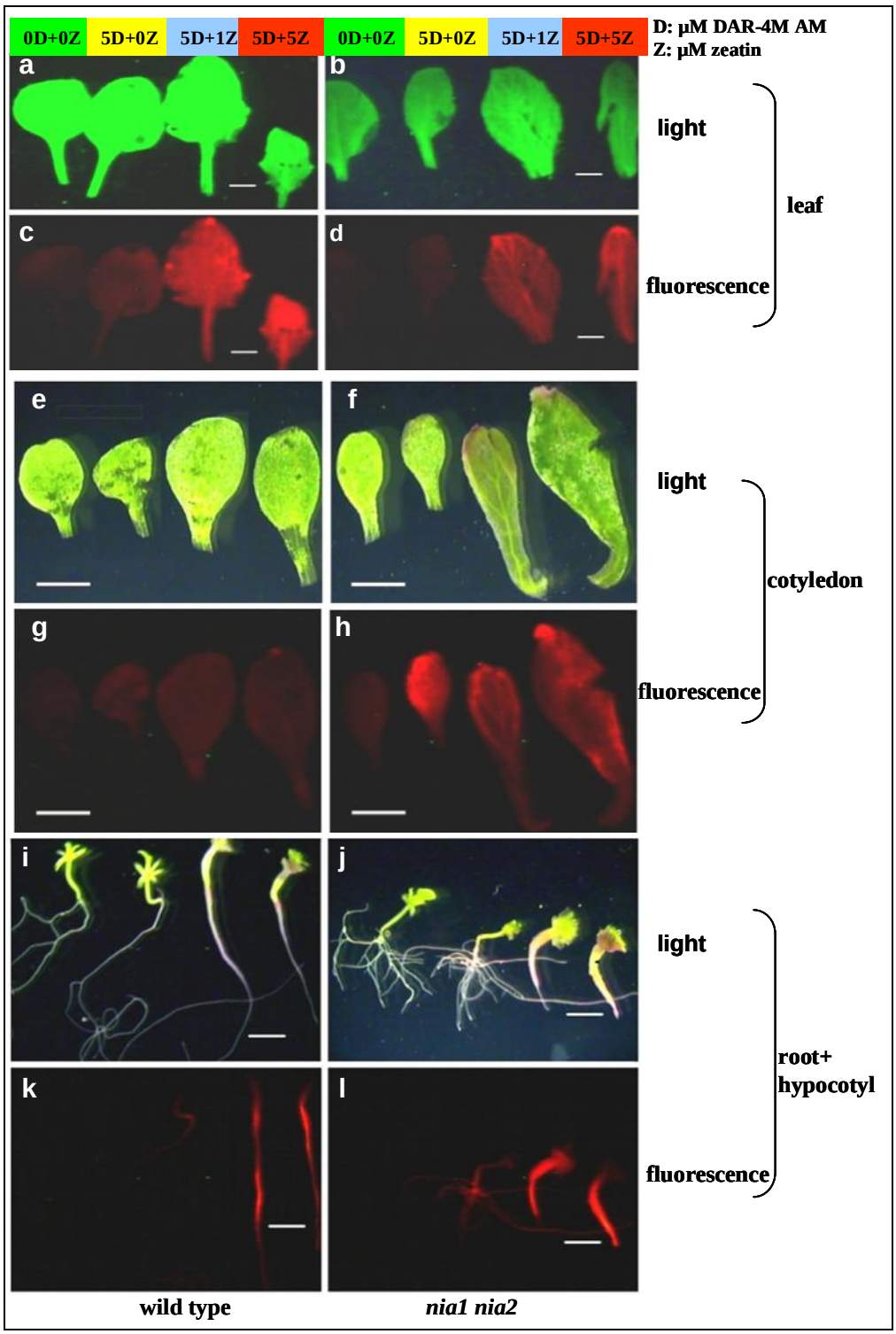


Fig. 8. Cytokinin-induced NO-dependent fluorescence formation in wild type and the *nia1 nia2* mutant *Arabidopsis* seedlings. The photographs (a, b, e, f, i and j) were obtained as light microscopy pictures while the photographs (c, d, g, h, k and l) were obtained as fluorescence microscopic-pictures of identical samples. The fluorescent images were taken by using filter set 14 with an excitation wavelength of 510-560 nm together with an emission wavelength of 590 nm. Bar = 1 mm.

3.2.2.2 Cytokinin-induced NO Formation and Inhibition of NO Formation by the Specific NO Scavenger PTIO in Wild Type *Arabidopsis* Seedlings

When seedlings were grown on MS agar containing DAR-4M AM for 14 days, the zeatin-dependent NO-fluorescence formation might be interfered with the dye uptake. Therefore, an experiment with a relatively shorter incubation time with zeatin and dye was performed. Moreover, dye and zeatin treatments were separated into two steps, the preincubation of the seedlings with dye first and then treatment of seedlings by zeatin alone, to exclude arguments that zeatin influences the dye uptake by the plant. Another purpose of this experiment was to demonstrate the fluorescence formation due to NO by using the NO-specific scavenger PTIO (Barchowsky et al., 1999). Seven days old wild type *Arabidopsis* seedlings were preincubated with 1 µM DAR-4M AM alone for 4 hours. Then seedlings were washed with double-distilled water and treated by zeatin with or without 1 mM PTIO for 2 days. The dark pictures of leaves (Fig. 9 c-f) which were not treated by DAR-4M AM showed that the autofluorescence of chlorophyll was effectively excluded. The zeatin-induced increase in NO-specific fluorescence was observed in leaves (Fig. 9 b and h), trichomes, (Fig. 9 h), the elongation zones of root tips (Fig. 9 j), and cotyledons (Fig. 9 p). A small effect of zeatin was observed in the root-shoot transition zones and the vascular bundles (Fig. 9 r). At the higher magnification, the accumulation of NO-dependent fluorescence could be observed mostly in vacuoles of leaf epidermal cells treated by 5 µM zeatin (Fig. 9 b). The cuticles of guard cells and a small vein lying underneath the epidermis cells were highlighted as an effect of zeatin treatment (Fig. 9 b). The vacuoles as acidic compartments accumulated the dye (Fig. 9 a and b) but probably also certain lipophilic substances like cuticles of guard cells and trichomes whereas during the 48 h treatment the guard cells themselves remained dark. It is inevitable that the resulting fluorescence after zeatin treatment could be a combination of intracellular distribution of dye and the tissue distribution of NO production. PTIO did not decrease completely DAR-4M AM fluorescence formation (Begum, 2005). The fluorescence formation was increased in root tips in the presence of PTIO (Fig. 9 m) and fluorescence in the leaves (Fig. 9 k) became more reddish. Therefore, this chemical had unexpected side effects on fluorescence formation at least in these parts of plant. The decrease of fluorescence formation by NO scavenger PTIO was apparent when plants organs

treated by zeatin and treatments with or without PTIO were compared (Fig. 9 h and i, Fig. 9 j and n, Fig. 9 p and t, Fig. 9 r and v). Therefore, the fluorescence formation was shown to be NO-specific.

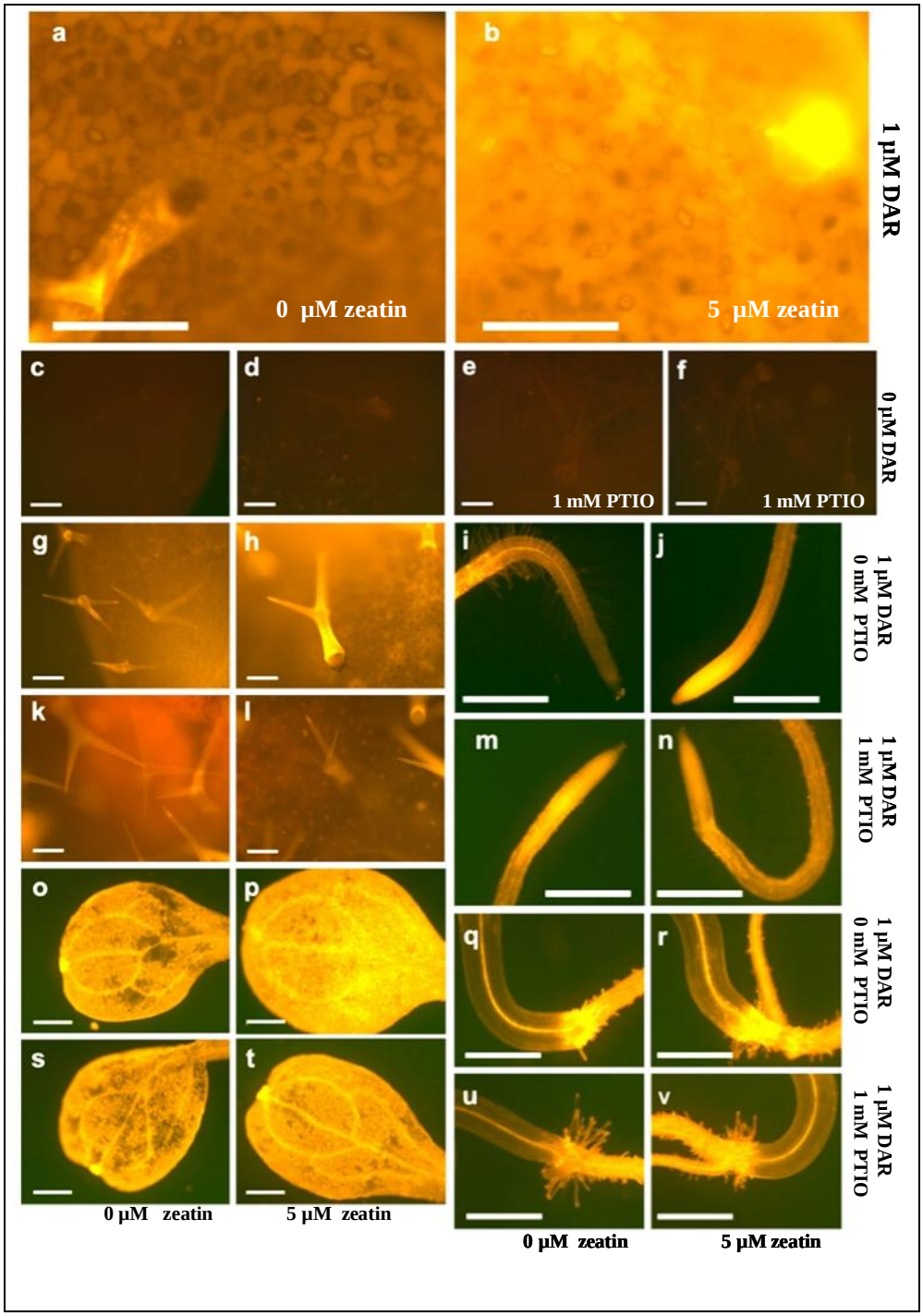


Fig. 9. Fluorescence micrographs of wild type seedlings treated by cytokinin and NO scavenger PTIO. Seven days old seedlings were preincubated 1 µM DAR-4M AM for 4 h. Then they were treated by 0 µM or 5 µM zeatin and 0 mM or 1 mM PTIO for 2 days. (a) and (b): leaf segment; (c), (d), (e), (f), (g), (h), (k) and (l): leaf; (i), (j), (m) and (n) root tip; (o), (p), (s) and (t): cotyledon; (q), (r) (u) and (v): root-shoot transition. The fluorescence microscopy pictures were recorded by using a filter set 20 with an excitation wavelength of 546/12 nm together with 575-640 nm emission. (a and b) Bar = 100 µm; (c-v) Bar = 500 µm.

3.2.2.3 Tissue Patterns of NO Formation in Wild Type and *Atnos1 Arabidopsis* Seedlings

During the course of this study, a new enzyme (*At*NOS1) for NO biosynthesis in plants was discovered by Guo and coworkers (2003).The involvement of *At*NOS1 in cytokinin-induced NO production was studied by using an *Atnos1* (*At*3g 47450) knockout line (gift from Prof. Dr. Nigel Crawford, San Diego, USA). The changes in patterns of NO production in the plant tissue and the morphological responses of these *Atnos1* knockout seedlings were compared to wild type (Fig. 10). The strong increase in fluorescence in the leaf due to zeatin was indistinguishable from that of wild type seedlings (Fig. 10 a, b, g and h). The responses of all other tissues (cotyledons and roots) were also observed in the same way as in wild type plants (Fig. 10 c, d, e, f, i, j, k and l). Therefore, it was concluded that this particular *At*NOS1 enzyme was not regulated by zeatin. As *AtNOS1* belongs to a small gene family of six homologous genes in *Arabidopsis*, the role of the other five genes and proteins in cytokinin-induced NO production remains to be investigated.

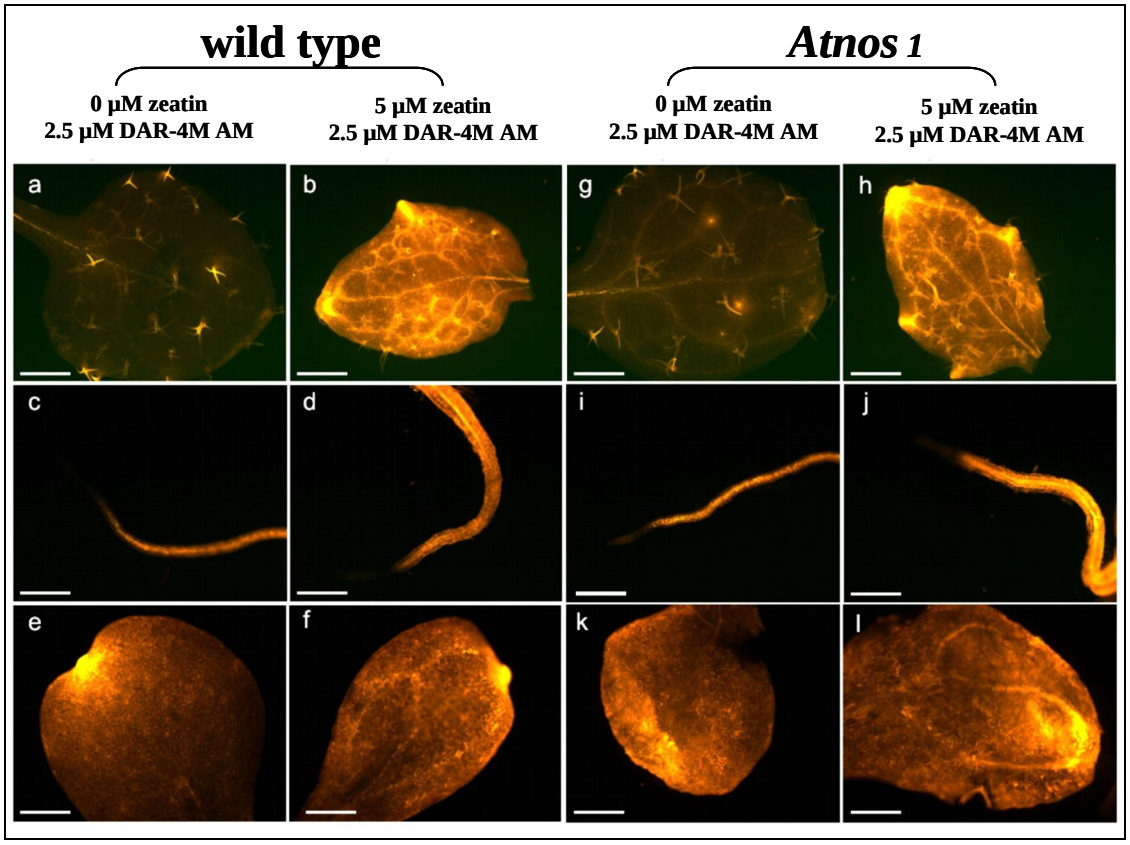


Fig. 10. Fluorescence micrographs of wild type and the *Atnos1* seedlings, grown for 14 days on solid MS medium containing 2.5 µM DAR-4M AM plus 0 µM or 5 µM zeatin. The fluorescence microscopy images were recorded by using a filter set 20 with an excitation wavelength of 546/12 nm together with 575-640 nm emission. Bar = 500 µm.

3.2.2.4 Comparison of tissue patterns of Cytokinin-induced NO Formation Patterns in Wild Type, *nia1 nia2* and *ahk4 Arabidopsis* Seedlings

After one of two possible enzymes proven to generate NO in plants was excluded as being a candidate enzyme in the cytokinin signal transduction by the experiment shown in Fig. 10, the role of the second potential plant NO-generating enzyme cytokinin action, NR, needed to be studied. An *Arabidopsis* cytokinin receptor *ahk4* knockout line (gift from Prof. Dr. Joe Kieber, UNC Chapel Hill, USA) was also studied. The roles of the NR-deficient mutant *nia1 nia2* and the *ahk4* in cytokinin-induced NO formation were investigated in the same experiment and compared to wild type by using the fluorescence microscopy method. In order to compare the tissue distribution of NO biosynthesis and the morphological changes of *Arabidopsis* seedlings in response to cytokinin, experiments with a long time of cytokinin treatment were carried out. Seedlings were grown for 14 days on solid MS medium

containing the dye and the increasing zeatin concentrations. The dye was transported throughout the whole seedlings and tissues, as the fluorescence micrographs showed. The zeatin concentration dependence of NO production was first studied in the wild type, the *nia1 nia2* double mutant (Wilkinson and Crawford, 1993), and in the *cre1/ahk4* knockout (Nishimura et al., 2004).

Tissue patterns of NO formation in the leaves of these three genotypes are compared in Fig. 11. In the micrographs of leaves, both effects of zeatin on the NO-dependent fluorescence formation and on the leaf morphology could be observed. When DAR-4M AM was omitted, no fluorescence formation was observed in the leaves (Fig. 11 d-f) while the images of the same leaves in light microscopy were green (Fig. 11 a-c). Therefore, the autofluorescence of chlorophyll was effectively cut off by the filter set 20 and did not obscure the results. The endogenous NO formation was observed mainly in veins and trichomes and hydathodes (Fig. 11 g-i). The zeatin effect was already strongly apparent at 0.5 µM zeatin (Fig. 11 k-n) and the NO-dependent fluorescence formation increased with the zeatin concentration (Fig. 11 k-t). Stomata or their cuticles, respectively, were highlighted as specks at low magnification (Fig. 11 k). The fluorescence formation in leaves of wild type, at 0.5 µM zeatin, was stronger than that of the *nia1 nia2* double mutant and the *ahk4* knockout (Fig. 11 k-n), and similar in all three genotypes at 5 µM zeatin (Fig. 11 r-t). The strongest growth inhibition by 5 µM zeatin was observed in leaves of the *nia1 nia2* seedlings (Fig. 11 r-t). All three genotypes exhibited the pointed tips at the leaves due to cytokinin treatment (Fig. 11 r-t).

Details of the leaves were resolved at higher magnification (Fig. 12). The increase of endogenous NO formation was clearly observed at a low zeatin concentration (0.5 µM) (Fig. 12 g-i). The increase of NO-dependent fluorescence formation also in the whole leaf blades (Fig. 12 g-q) and hydathodes (Fig. 12 m) became clearly apparent, besides trichomes and veins. The increase of NO-dependent fluorescence with increasing hormone concentration was apparent in all three genotypes.

Strong endogenous NO-fluorescence formation was observed in the cotyledons already of untreated *nia1 nia2* seedlings (Fig. 13 e). Fluorescence was enhanced already to the highest level by 0.5 µM zeatin (Fig. 13 h) and no further increase by the

higher zeatin concentrations was seen in this genotype (Fig. 13 l and o). Both endogenous and the zeatin-induced NO fluorescence formation in the cotyledons of the wild type and the *cre1/ahk4* seedlings were similar (Fig. 13 d, f, g, i, k, n, m and q). The cotyledons of all genotypes grew bigger due to the zeatin addition (Fig. 13). The details on the morphological responses are described later (Fig. 18 and Fig. 19 b).

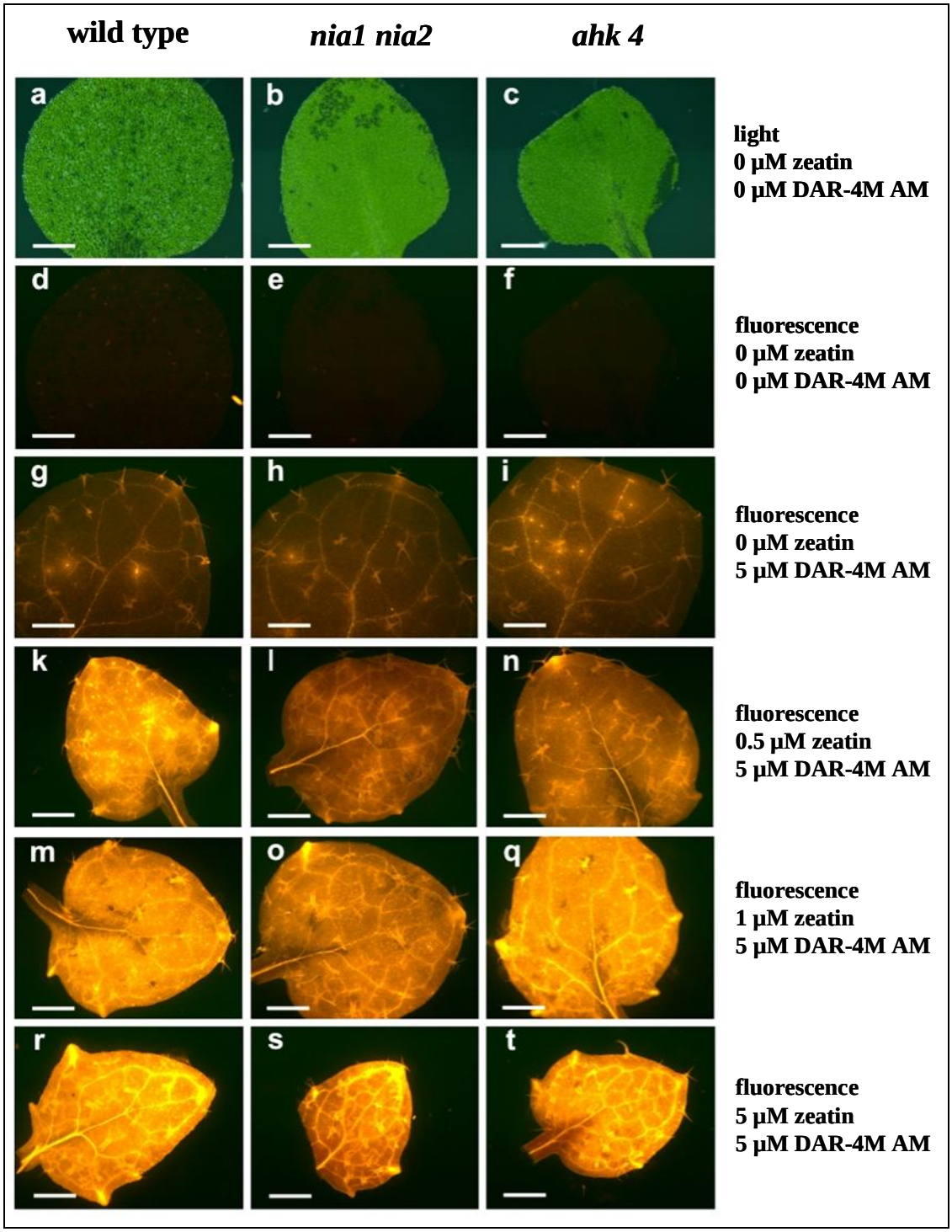


Fig. 11. Fluorescence micrographs of leaves of wild type, the *nia1 nia2* and the *ahk4* seedlings grown for 14 days on solid MS medium containing 0 µM or 5 µM DAR-4M AM and increasing concentrations of zeatin. The first row (a, b and c) shows the light microscopy images. The fluorescence microscopy images (d-t) were recorded by using a filter set 20 with an excitation wavelength of 546/12 nm together with 575-640 nm emission. Bar = 500 µm.

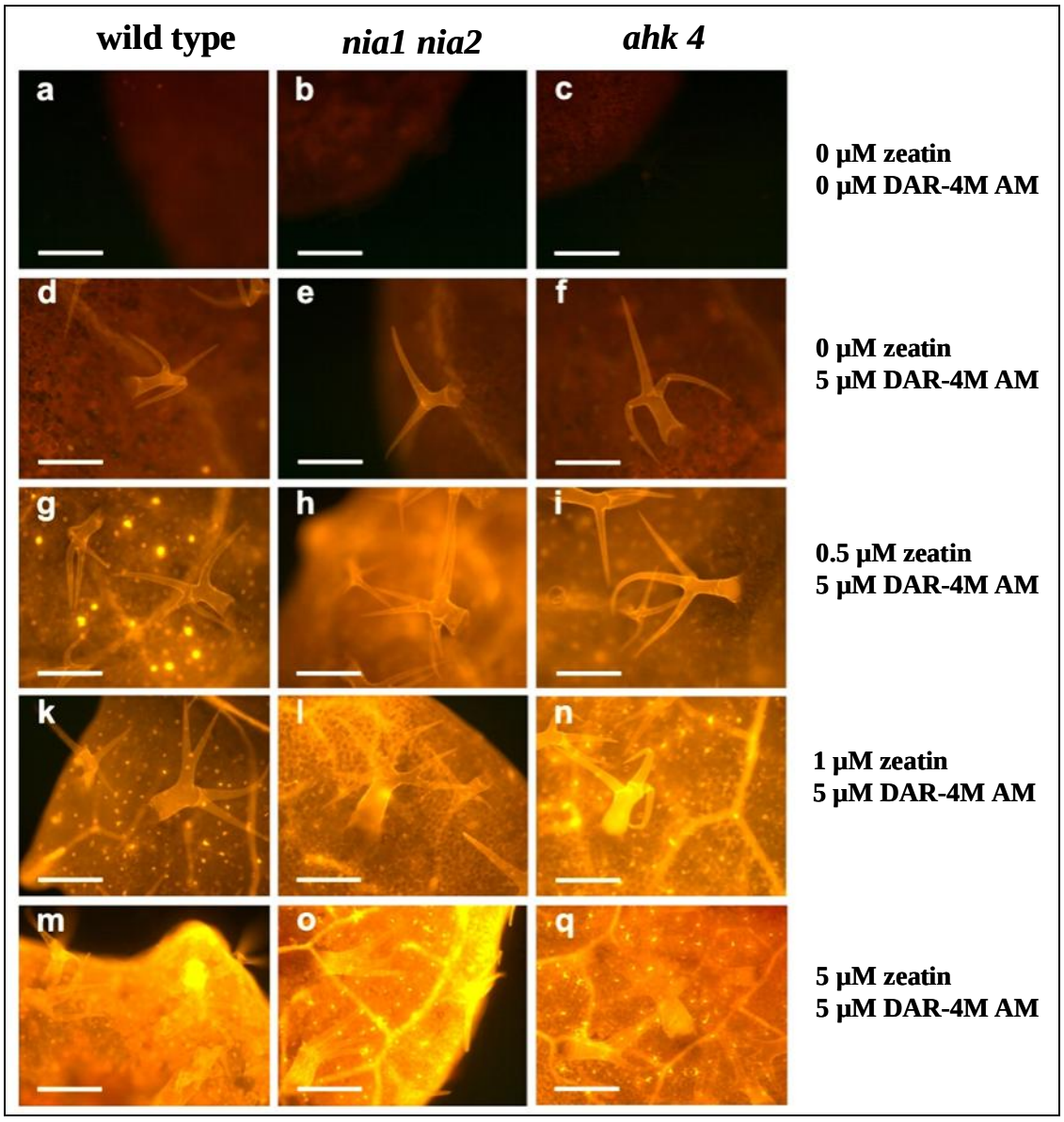


Fig. 12. Fluorescence micrographs of leaf segments of wild type, the *nia1 nia2* and the *ahk4* seedlings grown for 14 days on solid MS medium containing 0 µM or 5 µM DAR-4M AM and increasing concentrations of zeatin. The fluorescence microscopy images were recorded by using a filter set 20 with an excitation wavelength of 546/12 nm together with 575-640 nm emission. Bar = 100 µm.

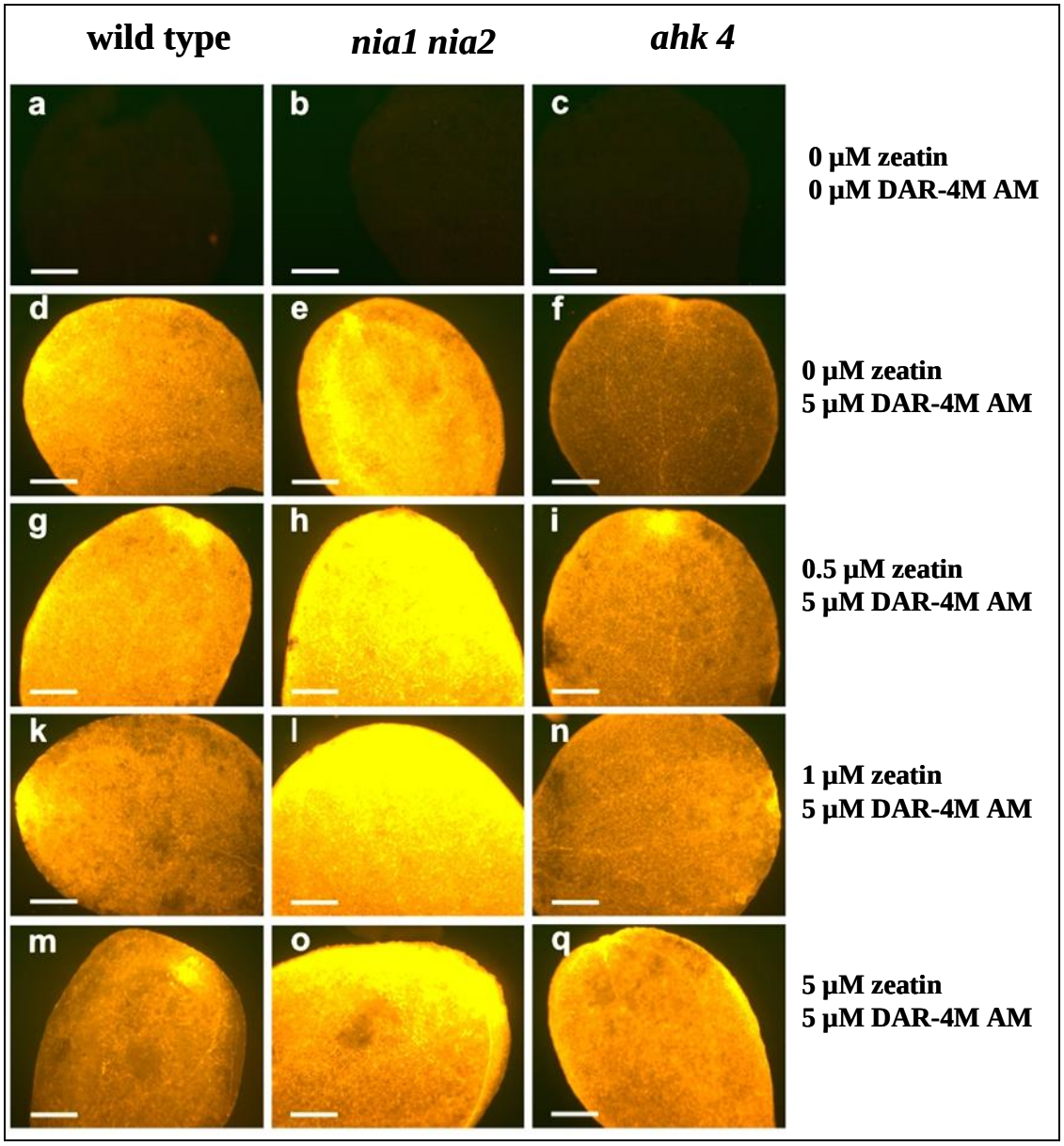


Fig. 13. Fluorescence micrographs of cotyledons of wild type, the *nia1 nia2* and the *ahk4* seedlings grown for 14 days on solid MS medium containing 0 µM or 5 µM DAR-4M AM and increasing concentrations of zeatin. The fluorescence microscopy images were recorded by using a filter set 20 with an excitation wavelength of 546/12 nm together with 575-640 nm emission. Bar = 500 µm.

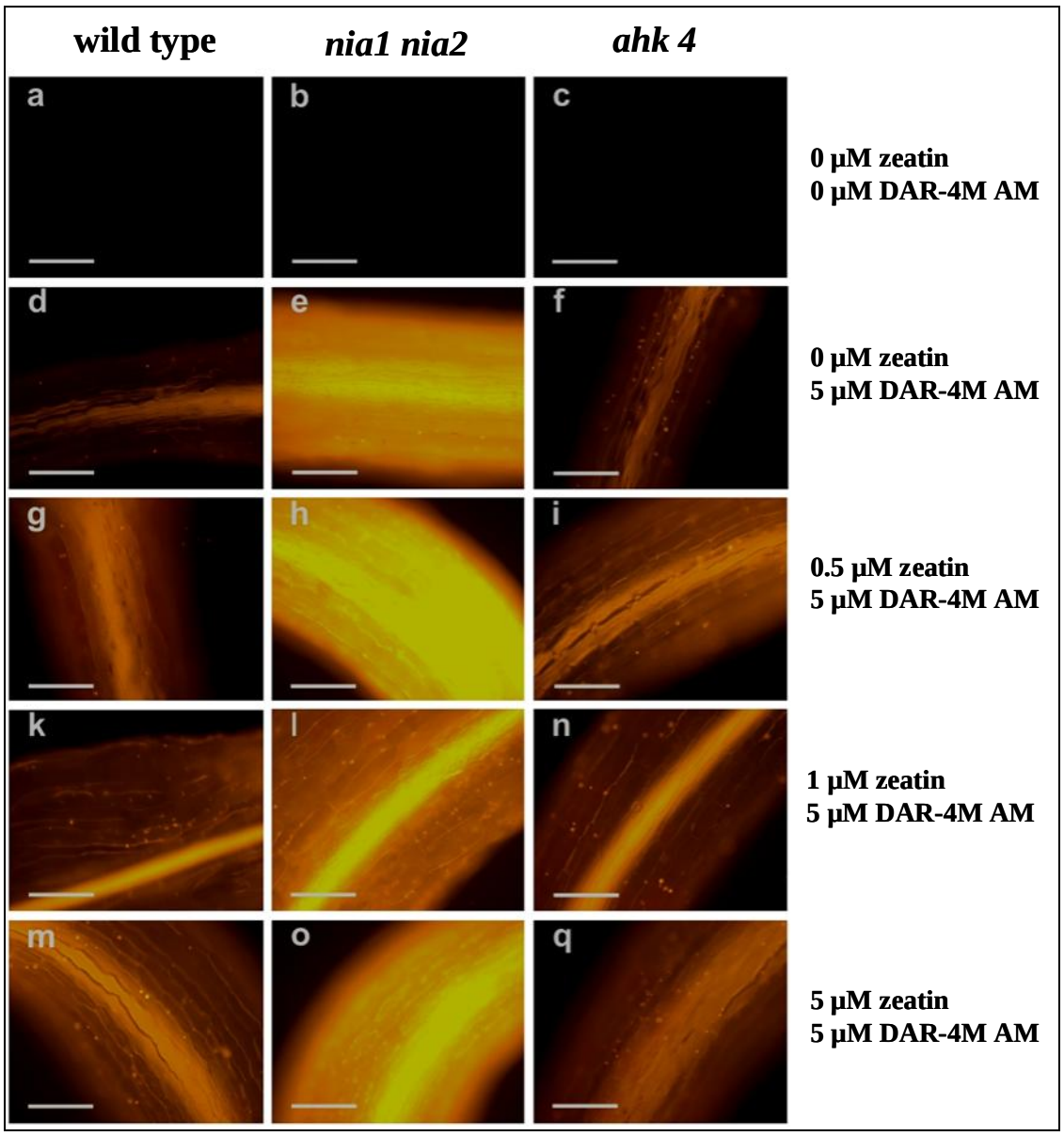


Fig. 14. Fluorescence micrographs of hypocotyls of wild type, the *nia1 nia2* and the *ahk4* seedlings grown for 14 days on solid MS medium containing 0 µM or 5 µM DAR-4M AM and increasing concentrations of zeatin. The fluorescence microscopy images were recorded by using a filter set 20 with an excitation wavelength of 546/12 nm together with 575-640 nm emission. Bar = 100 µm.

In hypocotyls of wild type and the *ahk4* seedlings, the endogenous NO production was observed mainly in the vascular bundles (Fig. 14 d and f). Fluorescence was increased by the addition of zeatin (Fig. 14 g, i, k, n, m and q). At high zeatin concentration, no further effect was observed (Fig. 14 k, n, m and q). The hypocotyls of the *nia1 nia2* seedlings exhibited strongly enhanced NO-dependent endogenous fluorescence in comparison to wild type (Fig. 14 e) and the effect of zeatin was seen at 0.5µM zeatin concentration (Fig. 14 h). No further increase in NO-dependent fluorescence by zeatin was observed (Fig. 14 l and o). Many bright spots were observed on the hypocotyls of the wild type and the *ahk4* seedlings (Fig. 14). They were located inside the parenchyma cells and not identified yet.

In the root tips, the meristems were highlighted and this fluorescence formation was further enhanced by zeatin in wild type and the *ahk4* plants (Fig. 15 d, f, g, i, k and n). However, in the root tips of *nia1 nia2* seedlings, endogenous NO production was already at a high level (Fig. 15 e) and no further effect by zeatin was observed (Fig. 15 h and l). Both the endogenous and the cytokinin-induced NO production in the root tip was observed in the meristematic region only, not in the root cap (Fig. 15). The root meristematic zone is the active cell division site where cytokinin has active function in plants (Taiz and Zeiger, 1998; Schmülling et al., 2003).

When the basal regions of roots of the three genotypes were studied in further detail, an increase in fluorescence in the stele and the cortex of the mature root in response to zeatin was found (Fig. 16). However, a small but remarkable difference was found in the vascular bundle of roots of the *ahk4* plants. In the zone of the mature roots, the *ahk4* seedlings did not accumulate as much NO-dependent fluorescence as the wild type seedlings (Fig. 16 k and n). The vascular bundle of roots is reported to be the site of strong expression of the AHK4 receptor (Mähönen et al., 2000; Nishimura et al., 2004). So that the lack of NO formation and AHK4 expression corresponded to each other.

In the *nia1 nia2* seedlings, strong fluorescence in the basal hypocotyls was observed already without zeatin addition (Fig. 16 e) which is in accordance with results of strong NO formation in hypocotyls (Fig. 14).

The strongest response of plants to zeatin to produce NO was observed mainly in the leaves (Fig. 13). Overall, wild type and the *ahk4* seedlings responses in leaves, cotyledons, hypocotyls and root tips were very similar whereas the *nia1 nia2* seedling responses were different as compared to wild type, especially in the hypocotyls, the cotyledons, and the root-shoot transition zones, where higher fluorescence was observed (Fig.11-16).

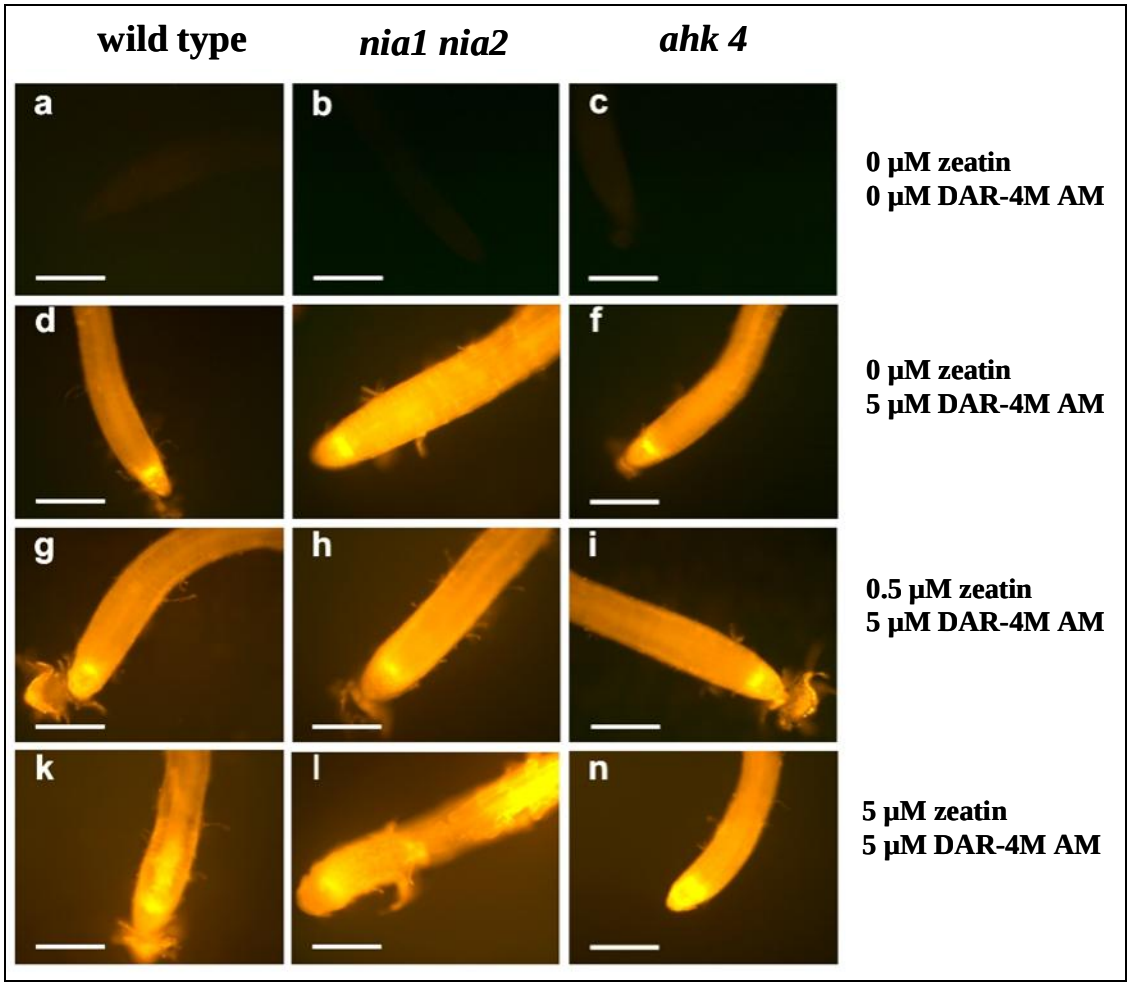


Fig. 15. Fluorescence micrographs of root tips of wild type, the *nia1 nia2* and the *ahk4* seedlings grown for 14 days on solid MS medium containing 0 µM or 5 µM DAR-4M AM and increasing concentrations of zeatin. The fluorescence microscopy images were recorded by using a filter set 20 with an excitation wavelength of 546/12 nm together with 575-640 nm emission. Bar = 100 µm.

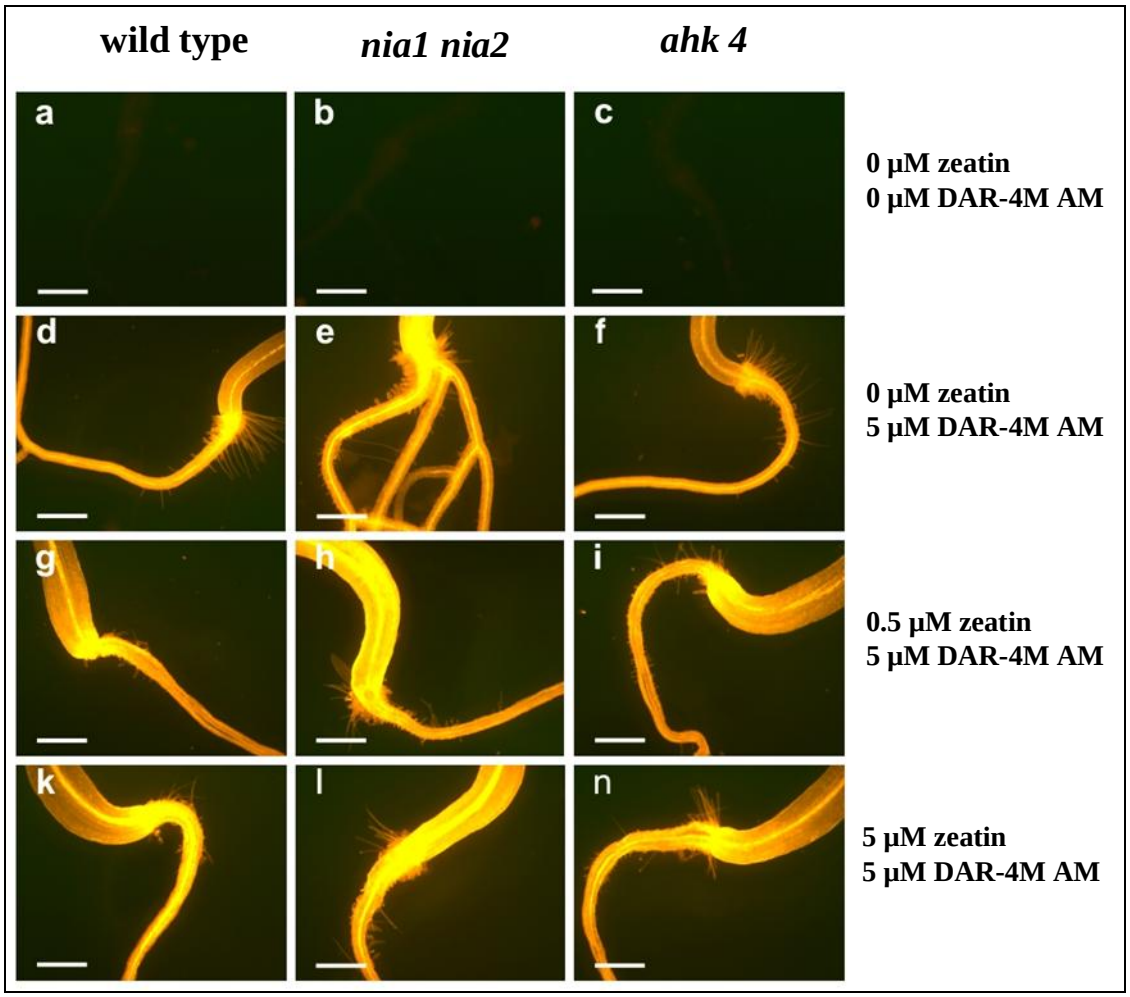


Fig. 16. Fluorescence micrographs of hypocotyls and root junction of wild type, the *nia1 nia2* and the *ahk4* seedlings grown for 14 days in solid MS medium containing 0 µM or 5 µM DAR-4M AM and increasing concentrations of zeatin. The fluorescence microscopy images were recorded by using a filter set 20 with an excitation wavelength of 546/12 nm together with 575-640 nm emission. Bar = 500 µm.

3.2.2.5 Different Patterns of NO Expression in Wild Type and *ahp1 ahp2 ahp3 Arabidopsis* Seedlings

In cytokinin signal transduction, the receptor histidine kinase transfers phosphate to AHP (histidine phosphate transfer protein) and then to ARR proteins. In the *Arabidopsis* genome five AHP genes are identified. If cytokinin-induced NO biosynthesis is dependent on functional AHP genes, a triple knockout like *ahp1 ahp2 ahp3* seedlings should show considerable defects in their NO response.

In both zeatin-treated and control *Arabidopsis* seedlings, the NO production in the leaf of the *ahp1 ahp2 ahp3* line showed similar a tissue pattern of NO production

compared to that of wild type seedlings (Fig. 17 a-d). The NO-dependent fluorescence formation response to zeatin was, however, stronger in the leaf of *ahp1 ahp2 ahp3* seedlings than in wild type seedlings (Fig. 17 b and d). Although the endogenous NO production without hormone in cotyledons of both genotypes was very similar, the cytokinin-treated cotyledons of the *ahp1 ahp2 ahp3* seedlings showed decreased in NO formation below the endogenous level (Fig. 17 e-h). In the vascular bundle of the main root, a strong NO-dependent fluorescence formation in response to zeatin was observed in wild type seedlings whereas no significant NO formation in response to zeatin was found in the *ahp1 ahp2 ahp3* seedlings. In the root tip, the same response pattern was observed (Fig. 17 i-p). Hence, although the endogenous NO production in both genotypes was similar, changes in NO generation in response to zeatin in the *ahp1 ahp2 ahp3* seedlings were observed (Fig. 17). Therefore a role of AHP proteins in a cytokinin-induced NO production in *Arabidopsis* is suggested here.

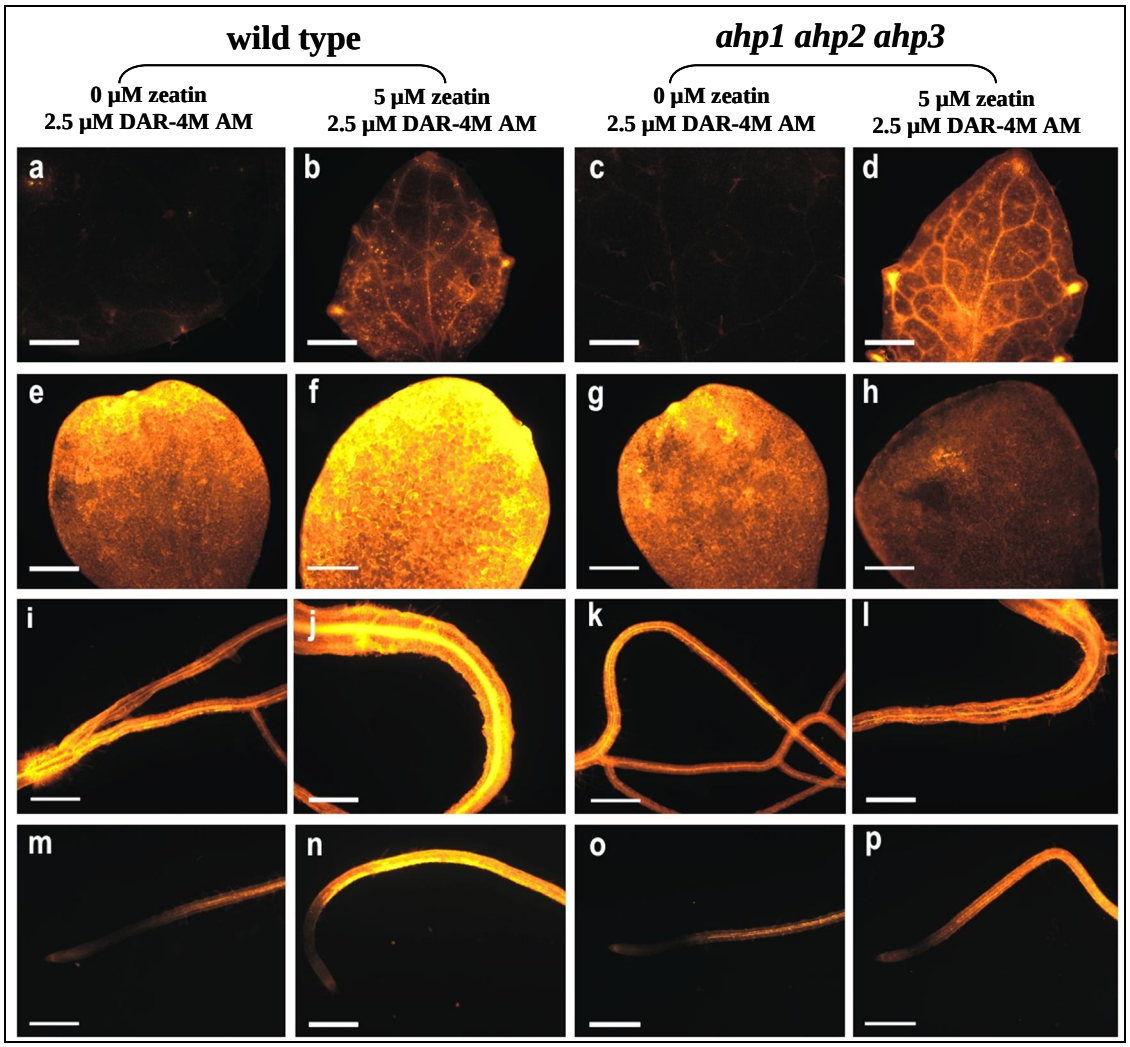


Fig. 17. Fluorescence micrographs of root of wild type and the *ahp1 ahp2 ahp3* knockout seedlings grown for 14 days in solid MS medium containing 2.5 µM DAR-4M AM plus 0 µM or 5 µM zeatin. The fluorescence microscopy images were recorded by using a filter set 20 with an excitation wavelength of 546/12 nm together with 575-640 nm emission. Bar = 500 µm.

3.2.3 Different Morphological Developments of *nia1 nia2*, Wild Type and *ahk4* Seedlings in Response to Cytokinin

The physiological responses to zeatin of 14 days old wild type, the *nia1 nia2* and the *ahk4* seedlings were studied by growing them on horizontal agar plates where the roots could grow into the agar (Fig. 18 and 19) in contrast to growth on upright plates (Nishimura et al., 2004; To et al., 2004). Growth of the *nia1 nia2* seedlings was already different without hormone in that the leaves were bigger (Fig. 18 a and Fig. 19 a). At 0.5 µM zeatin treatment the reduction of growth of primary leaves of the *nia1 nia2* already was dramatically visible (Fig. 18 a and Fig. 19 a) and leaves were comparable in size to that of wild type or the *ahk4* seedlings (Fig. 18 e-f and Fig. 19 a). The cotyledons of the mutant became larger and fleshier than that of wild type so that rather grotesque shapes originated (Fig. 18 g, j, h, k and Fig. 19 b). In addition, hypocotyls of the *nia1 nia2* became thicker and longer than that of wild type (Fig. 18 g, j and Fig. 19 c and d). Anthocyanin accumulation also was typically stronger in the *nia1 nia2* seedlings but depended also on the light (Fig. 18). The developmental responses of 14 days old wild type and the *ahk4* were almost identical (Fig. 18 and 19). The shorter root phenotype of the *ahk4* seedlings was observed only around 10 days after germination (Fig. 20 and 21 b) and this recovered after 14 days of germination (Fig. 21 a). These phenotypic recovery responses may be due to the overlapping functions of other AHK genes (AHK2 and AHK3) which are involved in cytokinin action.

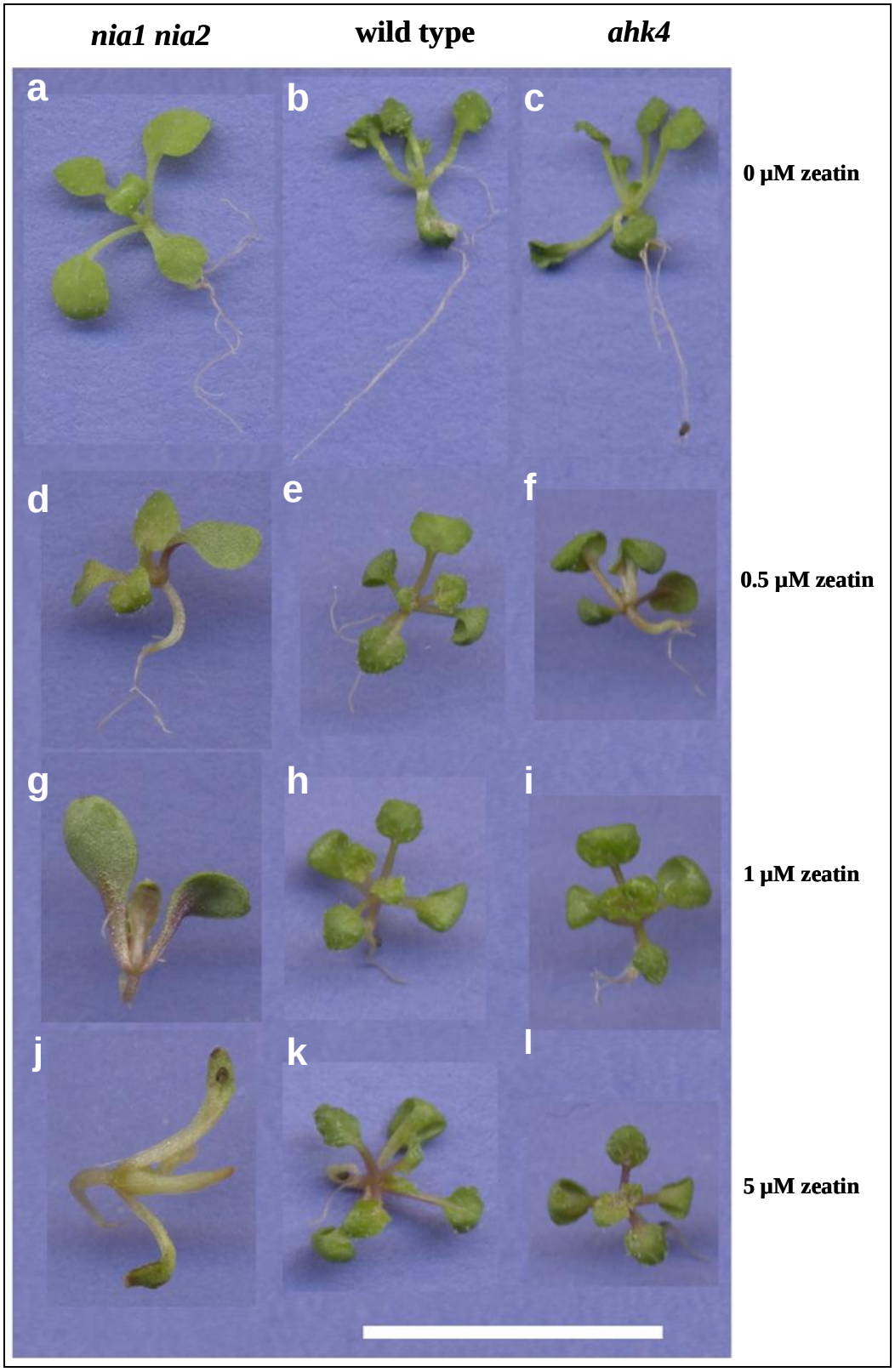


Fig. 18. Morphological responses of the *nia1 nia2*, wild type and the *ahk4*, seedlings to zeatin. The seedlings were grown for 14 days in solid MS medium containing the increasing zeatin concentrations. The images were taken by scanning at high resolution. Bar = 10 mm.

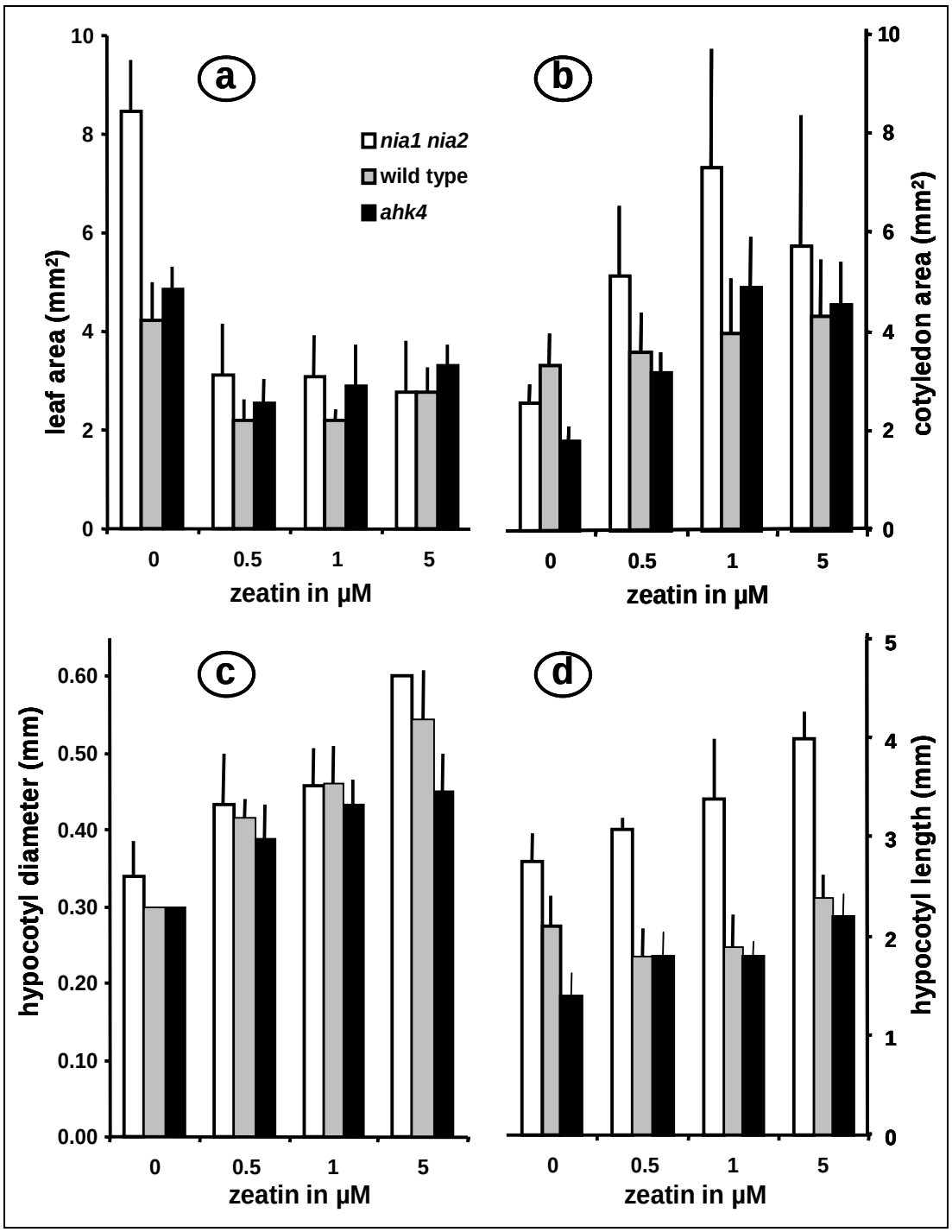


Fig. 19. Quantification of the morphological development of the *nia1 nia2*, wild type, and the *ahp4* seedlings in response to zeatin. The seedlings were grown for 14 days in solid MS medium containing the increasing zeatin concentrations. The morphological development of seedlings were recorded by scanning and quantified with an AxioVision Rel. 4.2 program. The data represented here are mean values and standard mean errors of 20-25 seedlings.

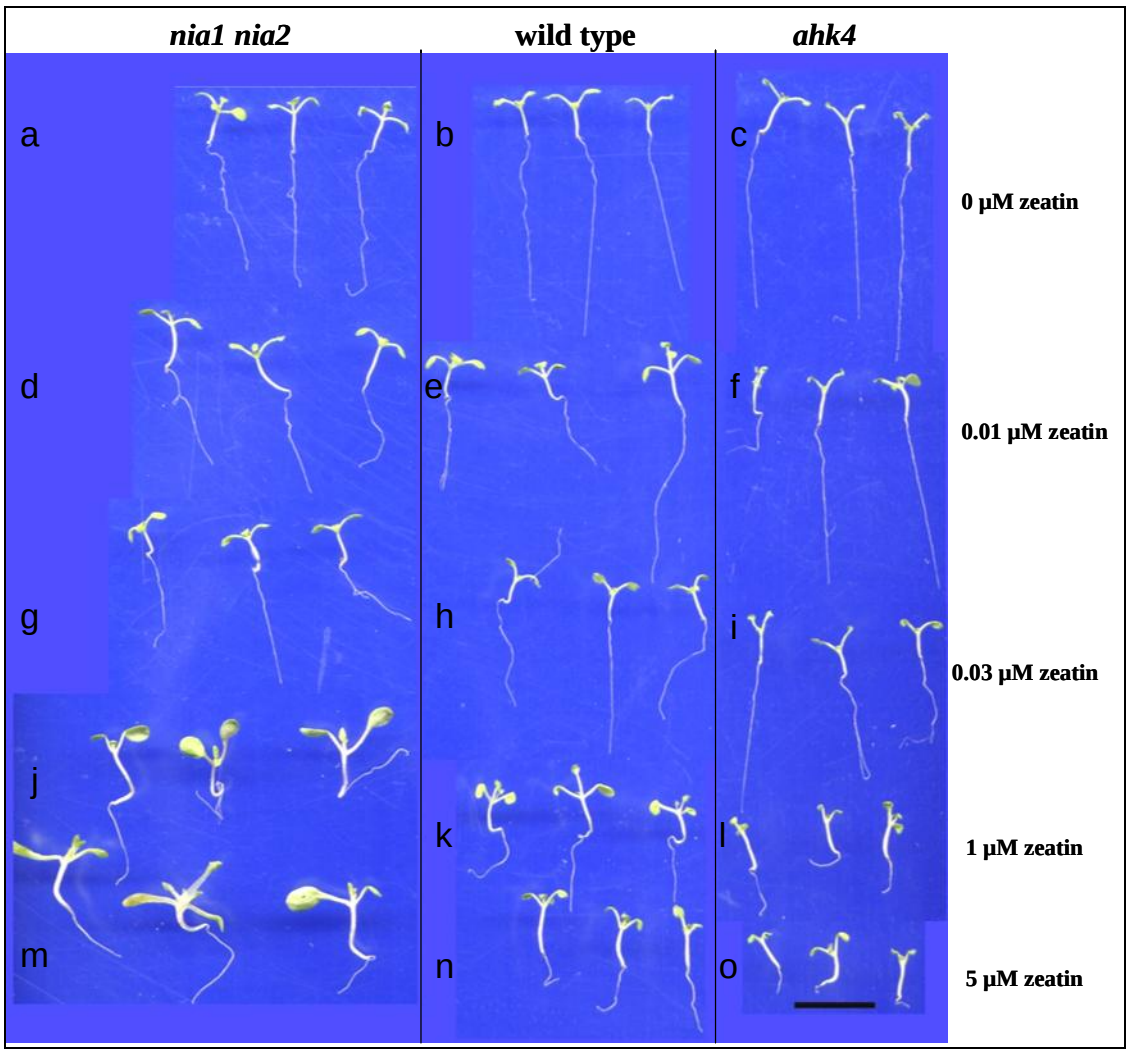


Fig. 20. Root development of 10-day old the *nia1 nia2*, wild type and the *ahk4*, seedlings in response to zeatin. The seedlings were grown for 10 days in solid MS medium containing the increasing zeatin concentrations. The images were documented by scanning at high resolution. Bar = 10 mm.

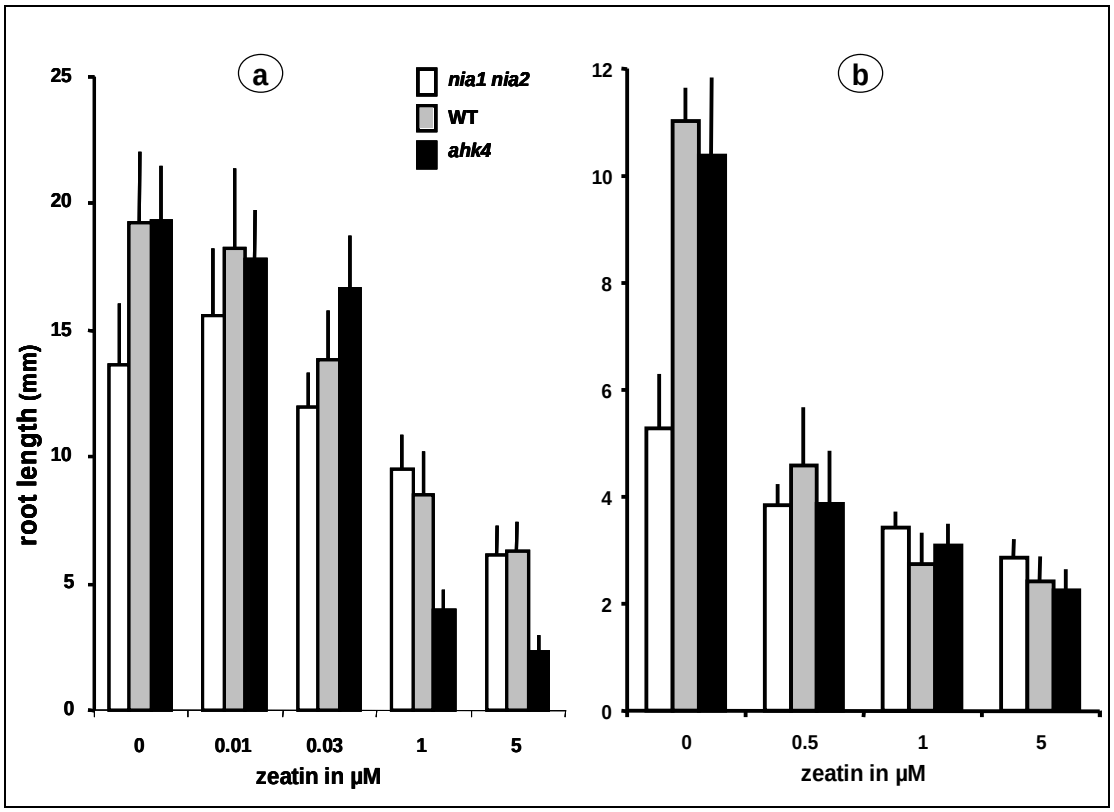


Fig. 21. Quantification of the root development of (a) 10 days and (b) 14 days old the *nia1 nia2*, wild type and the *ahk4* seedlings in response to zeatin. The seedlings were grown for 10 days and 14 days in solid MS medium containing the increasing concentrations of zeatin. The morphological developments of seedlings were recorded by scanning and measured with an AxioVision Rel. 4.2 program. The data represented here are mean values and standard mean errors of 20-25 seedlings.

3.3 Polyamines and NO Biosynthesis

3.3.1 Effect of Different Chemicals (PAs, Arg, SNAP, PTIO and H_2O_2) on DAR-4M Fluorescence Formation

In plants, PA degradation is carried out by diamine oxidase (DAO) and polyamine oxidase (PAO) (Bais and Ravishankar, 2002; Binda et al., 2002). DAO and PAO catalyse the oxidative deamination of biologically important amines with the formation of the corresponding aldehydes, ammonia, and H_2O_2 (Agostinelli et al., 2004; Sebela et al., 2001). H_2O_2 generation by Spm degradation is one of the ROS sources (Takahashi et al., 2003). Since hydrogen peroxide generated by polyamine oxidases might influence DAR-4M fluorescence, the reaction between H_2O_2 and DAR-4M in cell-free medium was checked. The effect of most commonly found PAs in plant cells such as Put, Spd, and Spm on DAR-4M fluorescence formation were tested. No effect of H_2O_2, Put, Spd, Spm and Arg (Fig. 22 and 23) on the DAR-4M fluorescence formation in the medium without plant cells was observed. Therefore, increase in fluorescence of DAR-4M in experiments with plant material and PA and Arg are due to NO and not disturbed by artifacts (Fig. 22 and 23). However, incubation with nitrite strongly increased DAR-4M fluorescence, an artifact, which is not mentioned in the literature (Desikan et al., 2002).

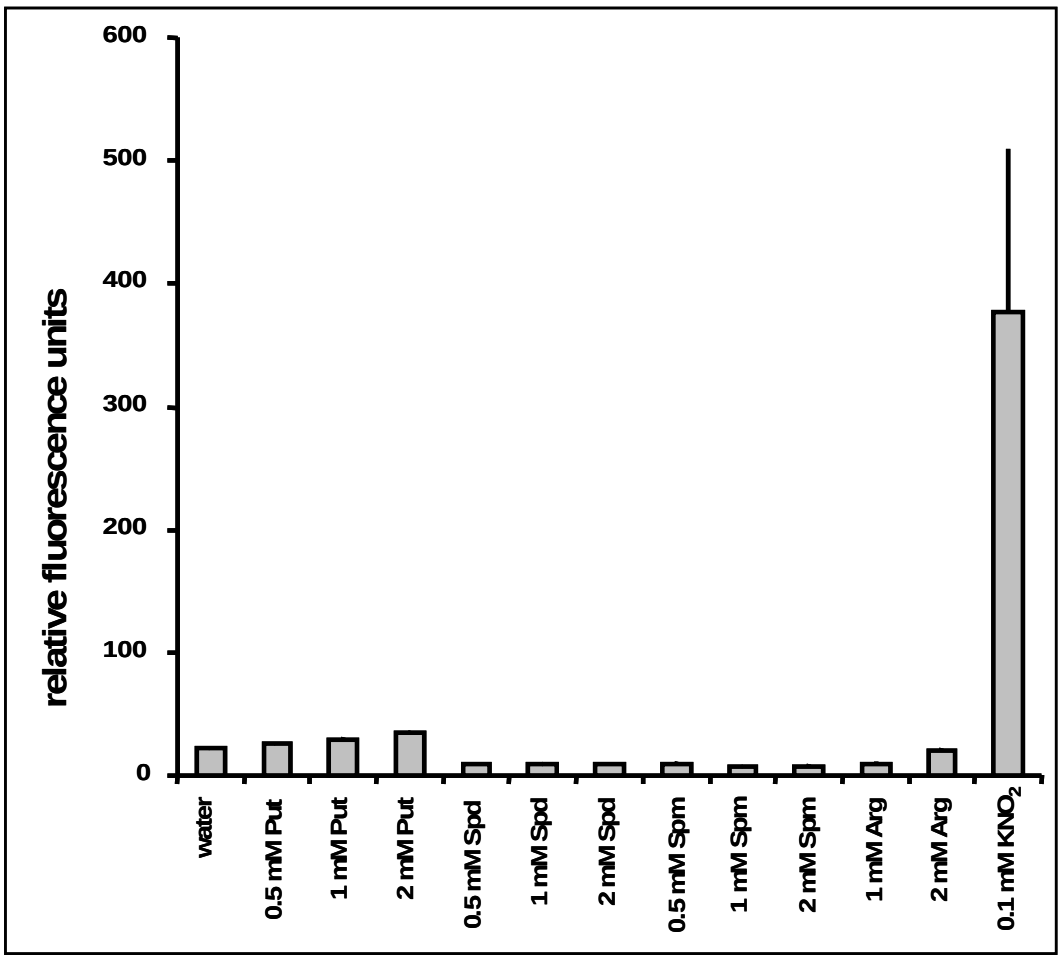


Fig. 22. Effects of chemicals on fluorescence formation of 2.5 µM DAR-4M. Different chemicals were incubated together with the fluorescent dye DAR-4M for 4 h in 30 mM MES buffer pH 5.7. The treatments were water, 0.5 mM Put, 1 mM Put, 2 mM Put, 0.5 mM Spd, 1 mM Spd, 2 mM Spd, 0.5 mM Spm, 1 mM Spm, 2 mM Spm, 1 mM Arg, 2 mM Arg and 0.1 mM KNO_2 as indicated in the figure. The relative fluorescence was measured with an excitation at 560 nm and an emission at 575 nm.

Incubations with plant cells showed that the inhibition of NO-dependent fluorescence by PTIO was only partial (Begum, 2005). Therefore it was necessary to check whether PTIO and DAR-4M compete for NO in cell-free media by using 0.2 mM SNAP, chemical donor of NO as a NO source (Fig. 23). The fluorescence increase induced by this NO donor could not be completely quenched by PTIO (Fig. 23), that competition of the regularly used concentration of 2.5 µM DAR-4M and 0.2 mM PTIO for NO might have caused incomplete inhibition of NO-induced fluorescence formation.

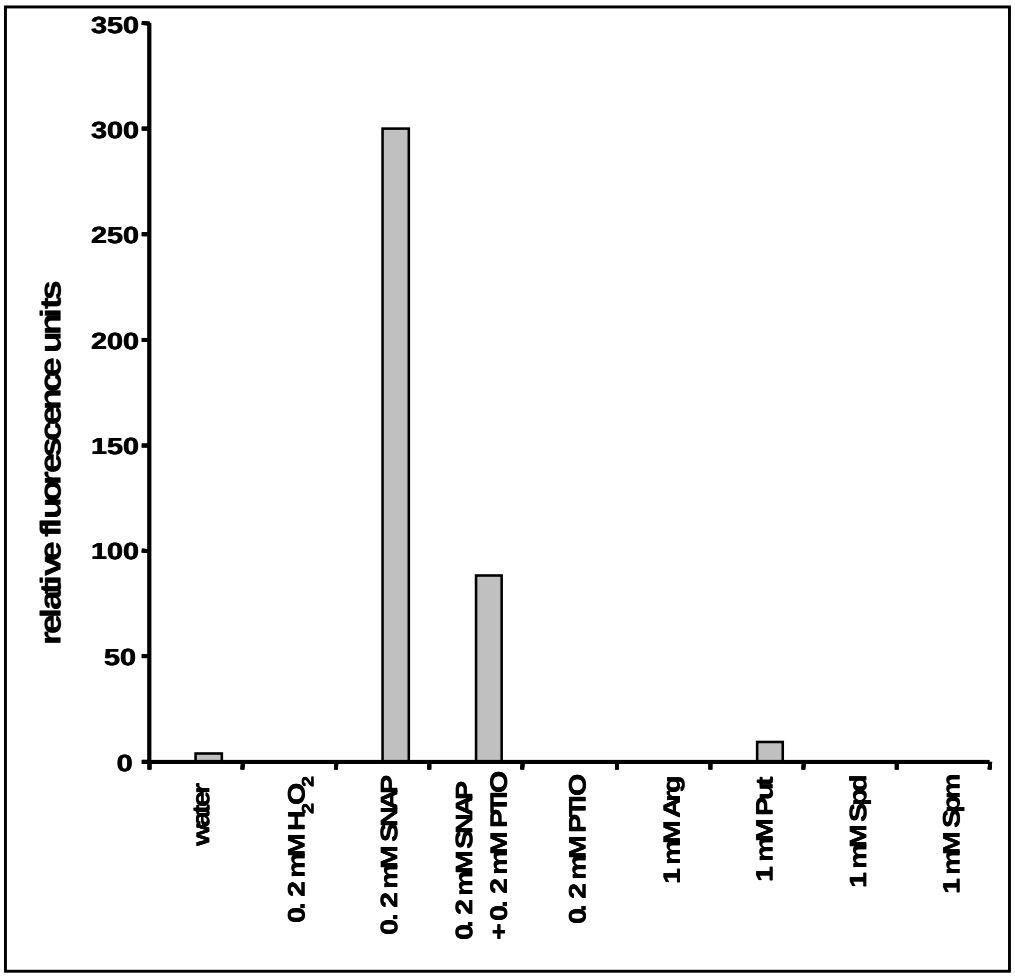


Fig. 23. Effects of chemicals on fluorescence formation of 2.5 µM DAR-4M. Different chemicals were incubated together with the fluorescent dye DAR-4M for 4 h in 30 mM MES buffer pH 5.7. The treatments were water, 0.2 mM H_2O_2, 0.2 mM SNAP, 0.2 mM SNAP + 0.2 mM PTIO, 0.2 mM PTIO, 1 mM Arg, 1 mM Put, 1 mM Spd and 1 mM Spm as indicated in the figure. The relative fluorescence was measured with an excitation at 560 nm and an emission at 575 nm.

3.3.2 PA-Induced NO Production in *Arabidopsis* Seedlings and *Nicotiana tabacum* Cells

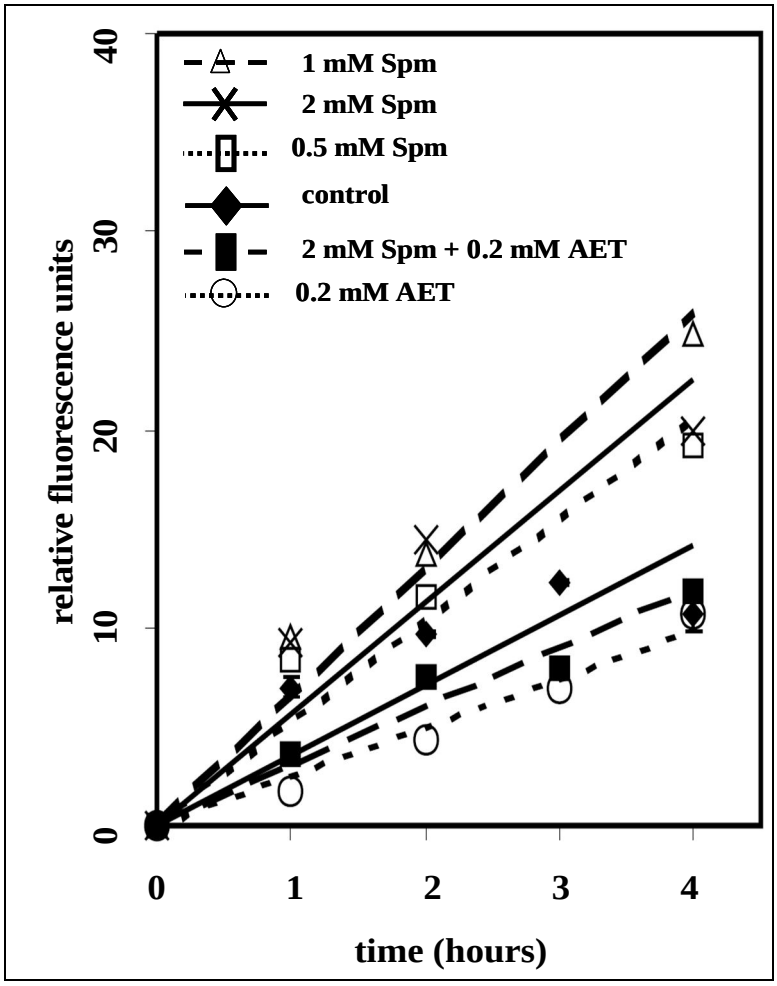


Fig. 24. Effect of Spm on NO release from wild type *Arabidopsis* seedlings and inhibition by AET. DAR-4M concentration was 2.5 µM. Lines were drawn as linear trend lines calculated by the program excel. The relative fluorescence was measured at excitation of 560 nm and emission of 575 nm.

The considerable increase in NO release by Spm was observed in wild type *Arabidopsis* seedlings (Fig. 24). Similar Spm-induced NO production was observed in tobacco BY2 cell culture (Fig 25). Spd was also an active compound to generate NO (Fig. 25) in tobacco cells and *Arabidopsis* seedlings (Fig. 26). However, the di-amine Put and animal NO precursor Arg has a little or no effect on NO production in both wild type *Arabidopsis* seedlings and tobacco cell cultures (Fig. 25 and 26). The non-biological amines tris and imidazole were inactive (Fig. 25). Although PA-dependence

of NO production in tobacco cells and wild type *Arabidopsis* seedlings was similar, NO production in *Arabidopsis* seedlings was less active in general.

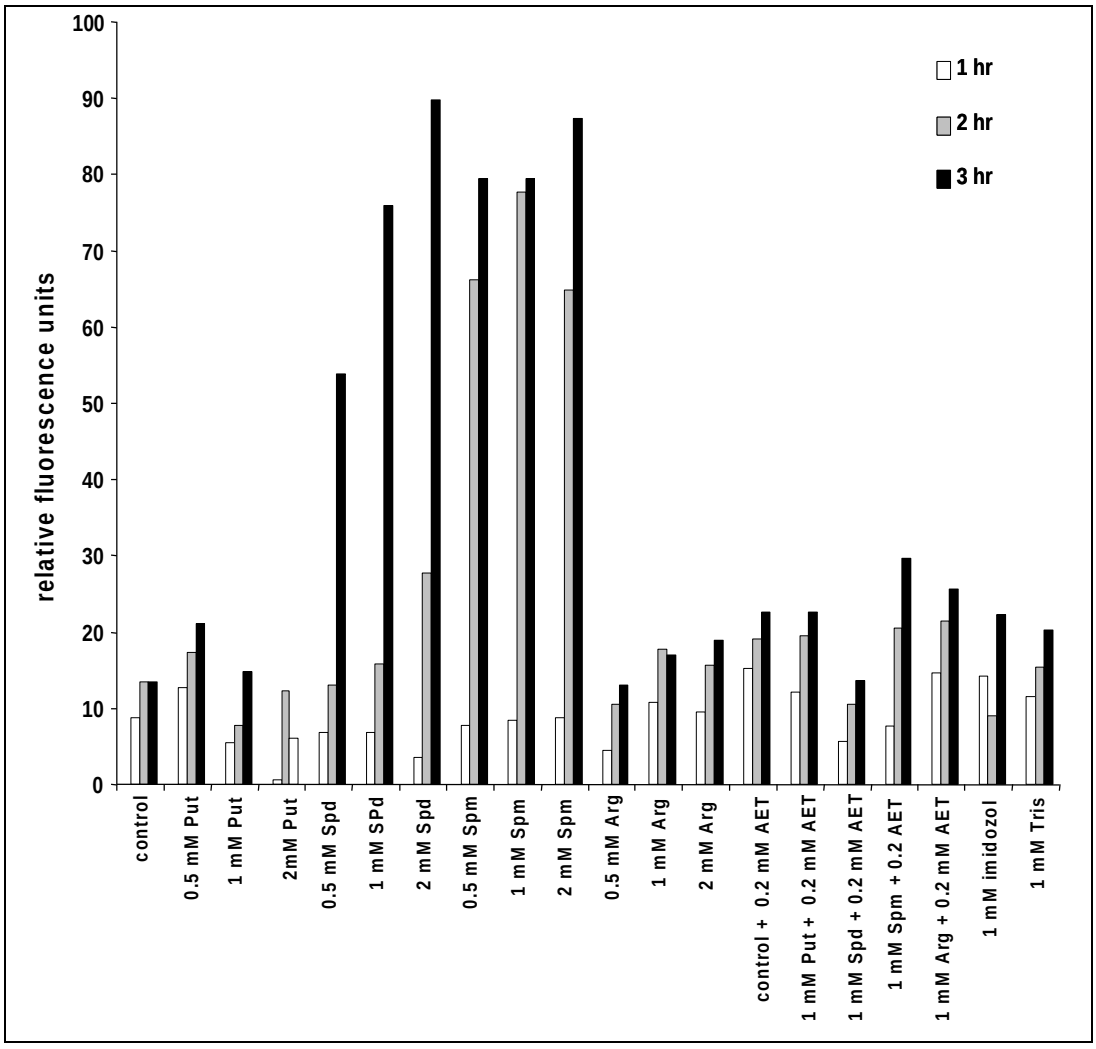


Fig. 25. Effect of Arg and different PAs on NO release in 6 days old tobacco BY-2 cells. Tobacco cells were incubated up to 3 hours with different concentrations of PAs or Arg plus 0 mM or 0.2 mM AET in the presence of 2.5 µM DAR 4M. The incubation condition was in the dark at 26 °C on a rotatory shaker (125 rpm). The relative fluorescence was measured at an excitation 560 nm and an emission at 575 nm.

PA-induced NO release was further characterized by a NOS inhibitor AET. AET which is an inhibitor of cytokinin-induced NO release (Tun et al., 2001; Carimi et al., 2005) inhibited both endogenous and NO release induced by PAs and Arg in *Arabidopsis* seedlings and tobacco cells (Fig. 24 and 25).

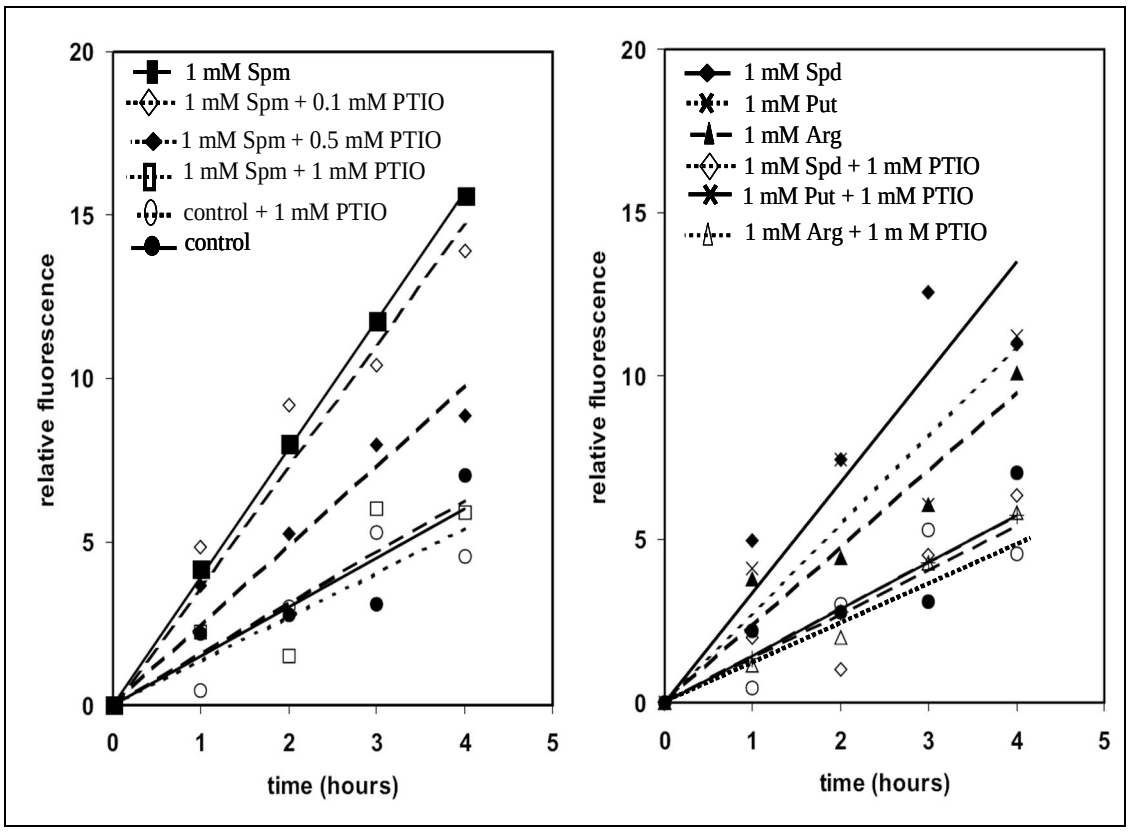


Fig. 26. Effect of PAs and PTIO on NO release from wild type *Arabidopsis* seedlings. DAR-4M concentration was 1 µM. The relative fluorescence units were measured with an excitation at 560 nm and an emission at 575 nm.

The specific scavenger for NO, PTIO, also decreased Spm-induced NO release in a concentration dependent manner (Fig. 26). Moreover, Spm-, Put- and Arg-induced NO production was decreased by addition of the NO scavenger PTIO to the incubation medium (Fig. 26). Endogenous NO release was only slightly decreased by PTIO. The concentration of DAR-4M was decreased to 1 µM (usually 2.5 µM) so that the effect of PTIO was stronger.

3.3.2.1 Time Dependent Spm –Induced NO Production in *Arabidopsis* Seedlings

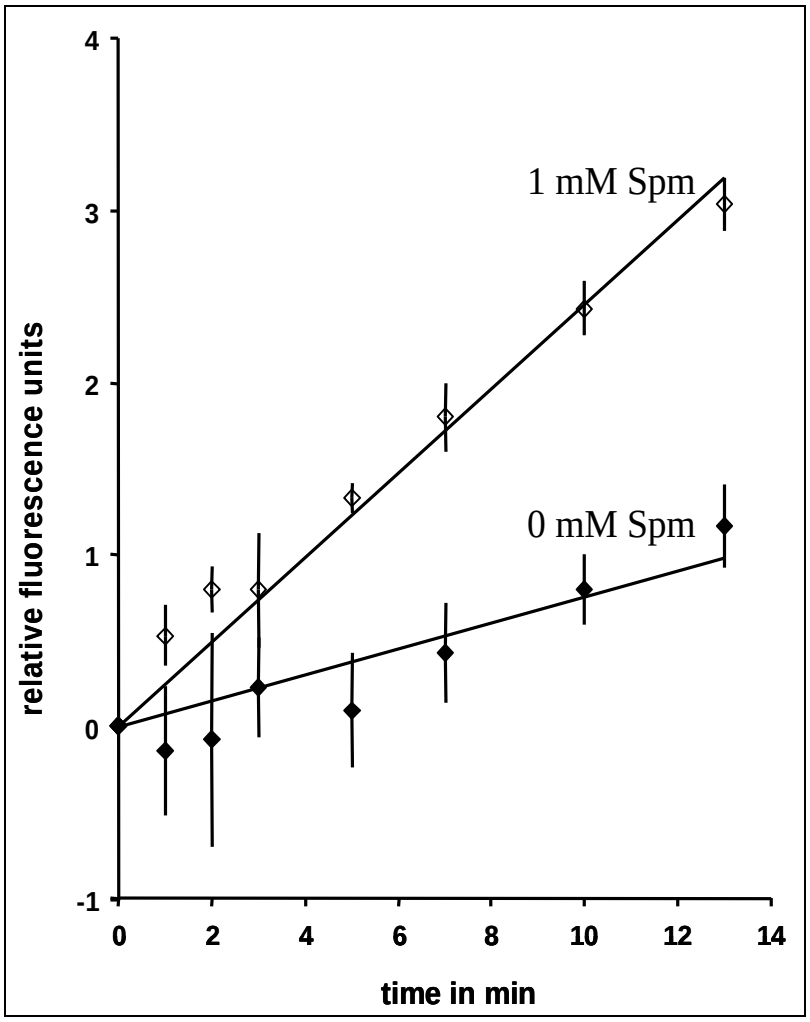


Fig. 27. Time dependence of NO release induced by 1 mM Spm from wild type *Arabidopsis* seedlings. The relative fluorescence was measured at an excitation at 560 nm and an emission at 575 nm. (n = 3; *SD*).

The time course of zeatin-induced NO fluorescence showed at three minutes lag phase (Fig. 4). It was an interest to compare this to the time course of PA-induced NO fluorescence. Therefore, the most active PA, Spm at 1 mM concentration, was used. Rapid release of NO above endogenous levels was observed when wild type *Arabidopsis* seedlings were treated with 1 mM Spm (Fig. 27). No lag phase could be found.

3.3.2.2 Tissue Pattern of PA- induced NO-dependent Fluorescence Formation

Although measuring the NO-induced fluorescence increase using DAR-4M in the medium showed clear relative fluorescence formation by PAs, it cannot show the tissue distribution of NO by PAs. Therefore, to analyze NO-induced fluorescence further, the fluorescence microscopy analysis of wild type *Arabidopsis* seedlings with the cell-permeable indicator DAR-4M AM was employed (Fig. 28). The potential interfering compound, H_2O_2, originating by PA conversion by PA oxidases did not increase fluorescence in a chemical test (Fig. 23). Spd- and Spm-induced NO-dependent fluorescence formation was at higher levels in the elongation zones of *Arabidopsis* root tips (Fig. 28) which is accordance with the data from Begum (2005). The seedlings treated with Arg showed no increase of NO formation in this tissue above the endogenous levels. Put had only a slight effect on NO-dependent fluorescence formation (Fig. 28). The data suggest tissue specificity of PA-induced NO biosynthesis in the wild type *Arabidopsis* root and specificity for the different PAs.

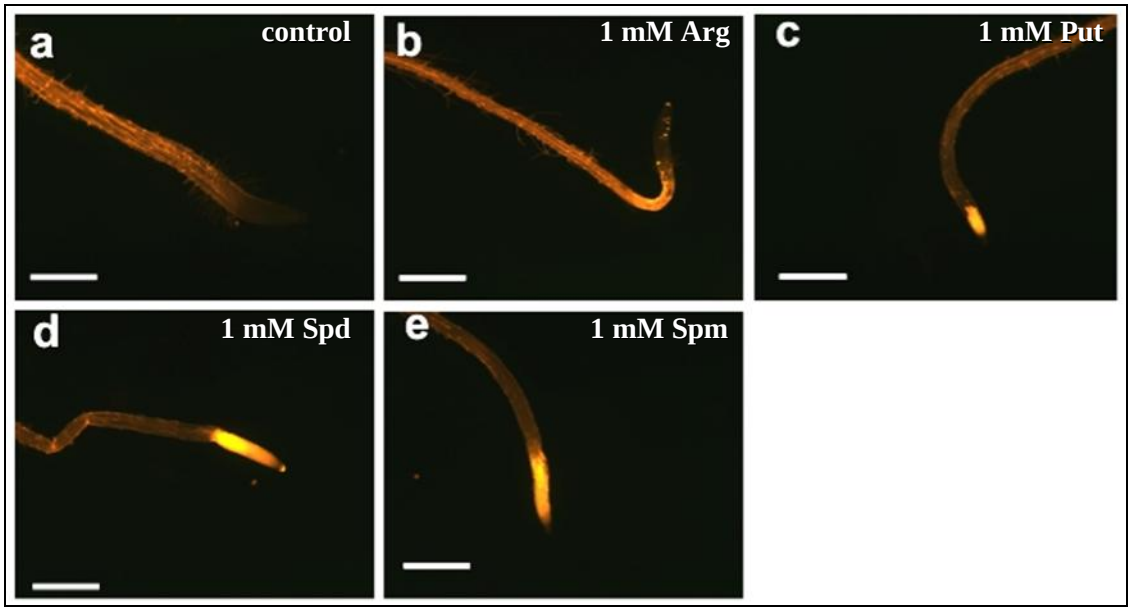


Fig. 28. Fluorescence microscopy of NO-dependent fluorescence formation in wild type *Arabidopsis* seedlings. Seedlings were pre-incubated with 1 µM DAR-4M AM in 30 mM MES buffer pH 5.7 for 4 h. Then *Arabidopsis* seedlings were treated by Arg or PAs in 30 mM MES buffer (pH 5.7) for 18 h. All micrographs were taken with the same camera setting. (a) no addition (b) 1 mM Arg (c) 1 mM Put (d) 1 mM Spd (e) 1 mM Spm. Bar = 500 µm.

3.4 Comparison of PA and Arg-Induced NO Released in Wild Type and *nia1 nia2 Arabidopsis* Seedlings.

PA-induced NO biosynthesis might be generated by the known enzymes of NO biosynthesis in plants, the *At*NOS1 (Guo et al., 2003) or by nitrate reductase (Harper, 1981). Therefore wild type and the *nia1 nia2* seedlings were compared in their responses to PAs treatment. NO production induced by PA in both wild type and the *nia1 nia2* seedlings was observed to be similar during the first three hours. Higher amounts of NO formation in the *nia1 nia2* line were observed after longer incubation times (4-24 h) (Fig. 29). Only Spd and Spm had a slightly increasing effect but not Arg during this time span. After 24 h, however, Arg slightly increased NO biosynthesis (by about 20%) whereas Spd and Spm strongly increased NO biosynthesis above control levels in both genotypes. The *nia1 nia2* line responded still slightly more than wild type seedlings. This indicated that NR is not the enzyme responding to PA addition with increased NO biosynthesis (Fig 29).

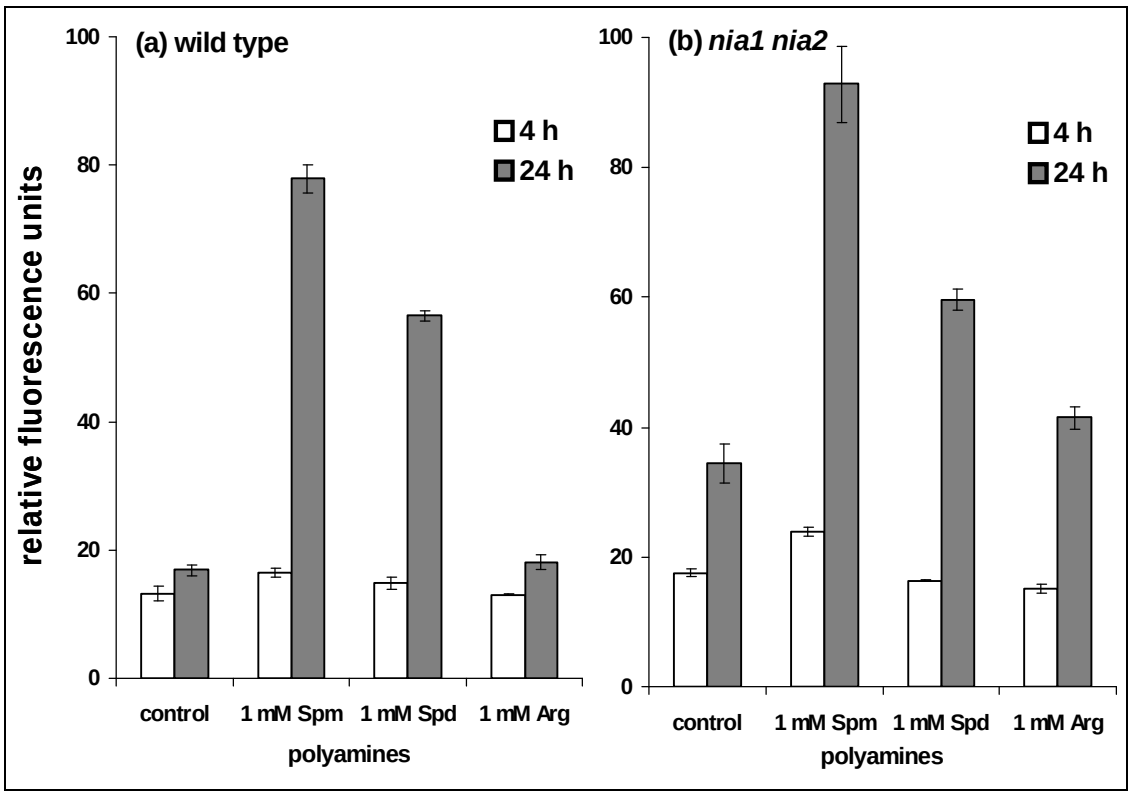


Fig. 29. Comparison of NO released by PAs and Arg in WT (a) and the *nia1 nia2* (b) *Arabidopsis* seedlings. *Arabidopsis* seedlings were treated with 1 mM PAs or 1 mM Arg in the presence of 1 µM DAR-4M. The relative fluorescence units were measured with an excitation at 560 nm and an emission at 575 nm.

4 DISCUSSION

4.1 Cytokinin Signal Transduction and NO Biosynthesis

4.1.1 Important Plant Nitric Oxide Biosynthesis Enzymes, Nitrate reductase and *At*NOS1, and Cytokinin-induced NO biosynthesis

Although NO is synthesised by a well characterized NOS in higher animals, NO biosynthesis in plants is not well understood yet. Several enzymes as sources of NO in plants have been reported, such as nitrate reductase (Harper, 1981), *Arabidopsis* NO synthase related to snail NO synthase (Guo et al., 2003), a membrane-associated nitrite reductase (Stöhr et al., 2003), a xanthine oxidase (Harrison, 2002). Moreover non-enzymatic sources of NO have been reported (Cooney et al., 1994; del Rio et al., 2004; Bethke et al., 2004). However their capability of rapid regulation and magnitude of contribution are not clear yet. NO was up-regulated within 2-3 min by elicitors in plants (Foissner et al., 2000; Lamotte et al., 2004; Zeidler et al., 2004) and by cytokinin in plant cell culture (Tun et al., 2001). Except for the nitrate reductase (Kaiser and Huber, 2001) little is known about rapid activity regulation of other NO biosynthetic enzymes. The *Arabidopsis* NO synthase (*At*NOS) activity was up-regulated by abscisic acid after 30 min (Guo et al., 2003). Here we observed a rapid NO production in both *nia1 nia2* and wild type *Arabidopsis* seedling by cytokinin arguing that NR might not be involved in the rapid regulation of NO biosynthesis by cytokinin. As a long-term contributor to the NO balance in plants NR is obviously necessary as absence caused an aberrant response to zeatin of *nia1 nia2* plants.

The observed high amount of NO production in the *nia1 nia2* seedlings, even it lacks one important plant NO synthesis enzyme, could be that nitrate is a substrate not only for NR but also for another NO-producing enzyme expect for NOS. In the *nia nia2* plants nitrate could be accumulated more than in the wild type and this accumulated nitrate could be used by other nitrate-using NO-producing enzymes. However, it has been reported that the double mutant line for both *NIA* (nitrate reductase) genes in *Arabidopsis* accumulated no more nitrate than the wild type and mutants had similar reduced nitrogen levels as compared to wild type (Wang et al., 2004). Further investigations should be carried out to understand the cause for having higher

endogenous NO levels in the *nia1 nia2* mutant seedlings.

Several inhibitors of the animal NOS were tested in many plant experiments. The direct effect of L-NAME (N^G-Nitro-L-arginine Methyl Ester, Hydrochloride) an isolated plant enzyme was shown on the plant NO synthase (Guo et al., 2003; Lindermayr et al., 2005). Other inhibitors used in vivo were L-NMMA (N^G-Monomethyl-L-Arginine, Monoacetate Salt) (Graces et al., 2001; Foissner et al., 2000) and AET (Tun et al., 2001; Carimi et al., 2005), all mimicking Arg or the guanino group of Arg. Arg mimicking compounds do not inhibit NO biosynthesis by NR (Rockel et al., 2002; Clarke et al., 2000; Sakihama et al., 2002). Therefore, inhibitor effects on NO biosynthesis must be attributed to the other NO synthesizing enzymes. Since AET inhibited cytokinin-induced NO biosynthesis in wild type and the *nia1 nia2* seedlings, a direct involvement of NR in the zeatin response could be excluded.

No change in the zeatin-induced NO-dependent fluorescence in the *Atnos* knockout seedlings was observed. So that this particular NOS does not seem to be involved in the cytokinin activated NO biosynthesis. Since five more genes of this *NOS* family are present in *Arabidopsis*, the role of the remaining NOS enzymes in cytokinin-induced NO production need to be investigated in future.

4.1.2 Tissue Distribution of Zeatin-Induced NO-Dependent Fluorescence and Components of Cytokinin Signaling Expression Patterns

Using the cell-permeable dye DAR-4M AM, NO formation patterns in *Arabidopsis* could be analyzed. The contributions of different tissues in NO biosynthesis and the differences in NO production patterns between wild type, the *nia1 nia2*, the *ahk4*, the *ahp1 ahp2 ahp3* and the *Atnos* knockout seedlings were studied.

The tissue distribution of zeatin-induced NO-dependent fluorescence showed a characteristic pattern which is especially striking in leaves where veins, trichomes, and hydathodes light up. Vascular bundles, root bundles, root tip meristems, and in shoot tips of young leaf tissues showed increased fluorescence by cytokinin. The cortical tissues in roots, hypocotyls and all leaf and cotyledon tissues also showed enhanced NO activity due to zeatin to a weaker extent. Similar expression patterns of known

components of the cytokinin signal transduction or action have been reported. The three cytokinin receptors (AHK2, AHK3, AHK4) expressed in meristematic tissues of shoot, root tips and growing leaf and lateral root primordia. Strong expression of AHK2, AHK3 and AHK4 are found in the vascular tissues (Nishimura et al., 2004). NO dependent fluorescence formation in the plants was also similarly observed in meristematic and vascular tissues in this study.

In addition, the tissue patterns of zeatin-induced NO-dependent fluorescence formation in veins and hydathodes were similar to the expression pattern of the type-A and type-B response regulator ARR genes which are involved in cytokinin signal transduction (Mason et al., 2004; To et al., 2004).

Type-A *Arabidopsis* response regulators (*ARRs*) are a family of 10 genes that are rapidly induced by cytokinin and act as negative regulators of cytokinin signaling. *ARR3* and *ARR4* are expressed mainly in the shoot and root vascular tissue, ARR5 is expressed in the shoot and root meristematic region, in all tissues in the root and in hypocotyls-root junction. *ARR6* expression is found in the shoot meristematic region, in cotyledon vasculature, in the hypocotyls, and in root tissue but not in the root tip. *ARR8* and *ARR9* are strongly expressed throughout the root (To et al., 2004). Increased NO formations were observed in the same tissue in this study.

The types-B *ARRs* were reported to be expressed in trichomes (*ARR2, ARR12* and *ARR19*), in leaf vasculature (*ARR1, ARR2, ARR10, ARR12, ARR13* and *ARR20*), in hydathodes (*ARR1, ARR2, ARR10, ARR12, ARR13* and *ARR20*), in young leaves (*ARR1, ARR2, ARR10, ARR11, ARR12, ARR13, ARR14* and *ARR18*), in shoot meristem (*ARR1, ARR2, ARR10, ARR11, ARR12, ARR13* and *ARR 20*), and in root tips (*ARR1, ARR2, ARR10* and *ARR12*) (Mason et al., 2004). These type-B response regulators expression patterns were similar to those tissue patterns of NO-dependent fluorescence as found in leaf vascular bundles, trichomes, hydathodes, root tips and young leaves of *Arabidopsis* seedlings in this work.

4.1.3 Cytokinin Oxidase/Dehydrogenase (CKX) and Isopentenyl Transfrase (IPT) Expression Patterns and NO-dependent Fluorescence Formation

Cytokinin oxidase/dehydrogenase (CKX) catalyzes the irreversible degradation of

cytokinins and is responsible for the majority of metabolic activity of cytokinin inactivation (Mok and Mok, 2001). In *Arabidopsis* the *AtCKX* gene family comprises seven members (*AtCKX1* to *AtCKX7*) (Schmülling et al., 2003). *AtCKX1* is expressed in the shoot apex, lateral shoot meristems, vascular bundle of roots, and the root-hypocotyls junctions. The expression of *AtCKX2* was detected in the shoot apex, and in stipules. *AtCKX4* is expressed strongly in trichomes, stomata, stipules and the root caps. So, *AtCKX5* expression was observed in the vascular cylinder within root apical meristem. *AtCKX6* expression is localized in the vascular system of cotyledons, leaves, and roots (Werner et al., 2003). *AtCKX* genes are expressed predominantly in the zones of active growth, where cytokinin, its substrate, is reported to be active. This is again a good correspondence to the tissues of cytokinin-induced NO-dependent fluorescence.

The expression patterns of zeatin-induced NO-fluorescence formation are also similar to those of the isopentenyl transferase genes (*IPT*), coding for the enzymes important for cytokinin biosynthesis. The expression of *IPT3*, *IPT5* and *IPT7* was found in veins of the leaves, stems and roots. *IPT5* was observed in the root meristem. *IPT7* was strongly expressed in trichomes (Miyawaki et al., 2004). Taken together NO might function in places of the high cytokinin activities in plants indicating its role in cytokinin signaling.

4.1.4 Aberrant Tissue Patterns of Zeatin-Induced NO-Dependent Fluorescence Correlated with Aberrant Zeatin Responses in *ahk4*, *ahp1 ahp2 ahp3* and *nia1 nia2* Seedlings

The aberrant tissue patterns of zeatin-induced fluorescence were observed in the mutant the *ahk4* and the *nia1 nia2* seedlings, which corresponded also to aberrant morphological responses of these genotypes to zeatin. In addition, the *ahp1 ahp2 ahp3* seedlings showed different patterns of tissue accumulations of NO formation in response to zeatin. The bundle of the *ahk4* root bases showed a diminished zeatin-induced fluorescence formation in comparison to wild type. In addition, a transiently stronger inhibition of root growth by zeatin was observed in ten days after germination of the *ahk4* seedlings. Since strong expression of *AHK4* was reported in this region of the young seedlings (Mähönen et al., 2000; Nishimura et al., 2004), the lack of AHK4

might have induced these changes in NO accumulation and in the morphology of *Arabidopsis* seedlings. This root growth inhibition was recovered when the *ahk4* seedling reached the age of 14-days. As two other cytokinin receptors, AHK2 and AHK3, are also expressed along the stele they may replace functionally the absent *AHK4* gene product in most developmental stages (Nishimura et al., 2004).

In both zeatin-treated and -untreated *nia1 nia2* seedlings, major changes in NO dependent fluorescence formation and morphological responses were observed in comparison to wild type. A strong increase of NO accumulation was observed in hypocotyls, cotyledons and the roots of the *nia1 nia2* seedlings even without any exogenous zeatin. The hypocotyls of the *nia1 nia2* seedlings treated by exogenous zeatin became thicker and longer than that of wild type. The leaves of the *nia1 nia2* plants with no zeatin treatment were expanded more. Upon zeatin addition, their sizes were reduced dramatically and comparable to the leaf size of wild type. Moreover, the cotyledons of the *nia1 nia2* seedlings, in comparison to wild type seedlings, were fleshier and already accumulated high NO-dependent fluorescence without any addition of exogenous zeatin.

Taken together, the balance of NO accumulation in *nia1 nia2* seedlings was disturbed and the absence of NR down-regulated one or several other NO generating enzymes in leaves and up-regulated such enzyme(s) in mature roots, hypocotyls and cotyledons, suggesting the regulation by NR on these enzyme(s). These NO producing enzyme(s), however, might not be NR since NO was rapidly released from the *nia1 nia2* seedlings upon zeatin addition and was also higher after longer incubation times. Moreover, the Arg analogue AET inhibition on NO production was observed in the *nia1 nia2* in the same way as in wild type. Taken together NR probably is not the candidate enzyme of rapid NO regulation by zeatin.

However, the other roles of NR in plant development should be taken into consideration here. NR as a source of NO and the changes in the *nia1 nia2* mutant seedlings in response to zeatin suggest a possible link between NO, NR and cytokinin. The observed physiological responses here may be linked to the long known interplay of cytokinin and the mutual regulation of NR and nitrogen metabolism by cytokinin (Taniguchi et al., 1998; Yu et al., 1998; Takei et al., 2001). Increase of nitrogen

sources up-regulates elements of cytokinin signal transduction (Sakakibara et al., 1998; Taniguchi et al., 1998; Kiba et al., 1999; Takei et al., 2001) and cytokinin up-regulates NR (Kende et al., 1971; Lu et al., 1990; Samuelson et al., 1995; Banowetz, 1992; Yu et al., 1998) have been reported by many observers. In addition the response regulator *ARR6* was induced by nitrate (Wang et al., 2000) showing the possible link between nitrate, NR and cytokinin signaling component. It will be interesting to see whether other known cytokinin response mutants show a relationship to NO biosynthesis (Chory et al., 1994; Deikmann and Ulrich, 1995; Faure et al., 1998; Vittorioso et al., 1998; Beemster and Baskin, 2000; Kubo and Kakimoto, 2000).

An aberrant pattern of NO accumulation in the zeatin-treated *ahp1 ahp2 ahp3* seedlings was observed in comparison to wild type seedlings. Without exogenous zeatin application, the NO accumulation in the *ahp1 ahp2 ahp3* seedlings was indistinguishable from that of wild type plants. When the seedlings were grown on zeatin-containing MS media, the NO-dependent fluorescence formation was missing in roots, hypocotyls, and cotyledons of the *ahp1 ahp2 ahp3* seedlings. The opposite response was observed in leaves. In conclusion, at least one out of three *AHP* genes was necessary for the plant for the correct NO-dependent responses to zeatin. One or more *AHP* genes might play a role in the upstream of regulation of NO biosynthesis by cytokinin.

4.1.5 Signal Transduction Models for Cytokinin Action and NO as Signaling Intermediate

In cytokinin signal transduction (Scheme 2, page 3), a biochemical signal is perceived by binding to histidine kinases which are receptors (AHKs), and transfer of the signal via the histidine phosphotransfer proteins (AHPs) to type-B response regulator proteins which act as transcriptional cofactors to regulate the expression of the type-A response regulators (Hutchison and Kieber, 2002; Kakimoto, 2003). However, this does not exclude other second messengers in cytokinin action besides this phosphorylation cascade as other proteins could be also phosphorylated. Moreover Kiba and coworkers (2005) have recently proposed a His à Asp phosphorelay-independent cytokinin pathway by showing that a certain number of genes were specifically and rapidly regulated by cytokinin similar to cytokinin primary type-A

response regulator gene *ARR5*.

Although the attempt to mimic cytokinin regulation of the type-A early cytokinin-induced *ARR5* promoter by NO donors failed (data not shown), there are nine more type-A *ARR* genes in *Arabidopsis* to be checked for their interaction with NO and NO inhibitors. It might also be the case that the cytokinin-induced NO signaling branches off from the mainstream of two-component cytokinin signal pathway after AHP phosphorylation and before type-B ARR functions. Therefore, the interaction between type-B ARRs and NO should be tested in the future to understand cytokinin-induced NO signaling.

The models of NO signaling in plants seem to be similar to animal models since the component of animal NO signaling such as cGMP (3´,5´-cyclic guanosine monophosphate) (Durner et al., 1999; Wendehenne et al., 2001) and calcium-activated channels (Gould et al., 2003; Lamotte et al., 2004), and cADPR (cyclic adenosine diphosphate ribose) (Allen et al., 1995) are necessary in NO signaling in plants. A cGMP independent NO signaling pathway was also reported in animal systems (Stamler et al., 2001). In this pathway, certain plasma membrane calcium channels are regulated by reversible nitrosylation by NOS without participation of cGMP (Stamler et al., 2001). An enzyme capable of removing nitrosylation at cysteines, to make nitrosylation reversible, was identified also in plants (Liu et al., 2001; Sakamoto et al., 2002) and proteomic profiling of nitrosylation was conducted (Lindermayr et al., 2005). The absence of one *AtNOS* gene did not show any difference in NO biosynthesis tissue patterns in compared to wild type. In addition, there are only a few overlapping genes expression by cytokinin (Rashotte et al., 2003) and NO (Polverari et al., 2003). At present, therefore, no clue is provided what protein might be regulated by cytokinin to induce NO.

Although the role of NO in cytokinin signaling is proposed here, how NO is involved in cytokinin signal transduction remains unclear. Many more experiments need to be done in order to confirm the hypothesis. It is necessary to check all possible reaction between cytokinin components and NO. Furthermore, it is also necessary to understand the enzymes responsible for cytokinin-induced NO biosynthesis.

Although data presented here could answer partially the role of NO in cytokinin signal transduction, it is still the beginning of new exciting field of NO and cytokinin signaling research.

4.2 Polyamines and NO biosynthesis

4.2.1 PAs Stimulated NO Biosynthesis and Role of Potentially Important Plant NO Generating Enzymes

The treatments by tetra amine Spm and the tri amine Spd showed a considerable increase in nitric oxide production in *Arabidopsis* seedlings and tobacco cell cultures while the di amine Put and their precursor Arg had no obvious effect. Several findings suggest that Put may have a different effect than those reported for Spd and/or Spm. Put has been reported to cause depolarization of membranes and increase in potassium leakage. Spd and Spm were shown to reduce ethylene synthesis by inhibition of ACC synthase (Bouchereau et al., 1999). The PA-induced NO production was quenched by addition of an NO scavenger (PTIO) and an inhibitor of the animal NO synthase (AET). These data suggest that the PA, Spd and Spm, might be involved in the NO biosynthesis in plants. Spm was observed to be the most active compound for NO production from *Arabidopsis* seedlings and tobacco cells. Although NO biosynthesis in animals is well characterized, NO biosynthesis in plants is still not clearly understood. No gene or protein which is similar to the large animal NOS has been found in plants. Therefore the biosynthesis of NO seems to be very different from that of higher animals. *Atnos1* has been reported recently as an *Arabidopsis* NOS which uses Arg as substrate but which is not similar to higher animal NOS (Guo et al., 2003). More NO synthesing enzymes and non-enzymatic NO production also have been reported (Bethke et al., 2004). Despite the evidence for the presence of NOS1, which uses Arg as a substrate in plants, some conflicting data, such as L-arginine as an ineffective substrate in green algae and no effect of arginine analogous NOS inhibitors on NO production in green algae and *Arabidopsis* cells were also reported (Mallick et al., 2000; Sakihama et al., 2002). Exogenously applied Arg was observed as ineffective for NO production in *Arabidopsis* seedlings and tobacco cells in this study. Therefore, the involvement of plant NOS1 in PA-induced NO biosynthesis is unclear.

NR is another important plant NO biosynthesis enzyme, which could generate NO as a side product (Harper, 1981). Higher NO production in response to Spm in the *nia1 nia2* double mutant seedlings than in wild type was observed in this study. This high

NO production level in the *nia1 nia2* seedlings in response to PA treatment argue against the role of NR in PA-stimulated NO biosynthesis.

As an alternative hypothesis, it can be assumed that either PAs were directly converted to NO by unknown enzymes, or upon breakdown of PAs through oxidative deamination or one of the reaction products of polyamine oxidase such as aminobutyladehyde or 1, 3-diaminopropane, was further converted to NO rapidly. Recently the possible role of PA oxidases in NO biosynthesis in *Arabidopsis* was reported (Begum, 2005). However, PA oxidases are not known to generate NO in animal systems. Plant PA oxidase might possess a different enzymatic mechanism (Sebela et al., 2001; Binda et al., 2002). Allan and Fluhr (1997) reported that PA oxidase using the substrate Put could be inhibited by L-NAME, a NOS inhibitor. Whether or not PAs regulate the plant NO producing enzymes, including NOS1 or NOS1-like enzymes, or PA oxidases, remains unclear.

4.2.2 NO Detection and Quantification by Fluorescence Dye

Ideally, NO should be measured accurately and reliably at its cellular site of action which means that any technique used to detect NO should measure NO and not another molecule, and not be prone to interference. In fact, the measurement of NO has proven to be difficult due to its short life span (3-5s) and high reactivity with other molecules (Kerwin et al., 1995). Therefore, a vast effort has been given to develop methods to monitor the intercellular dynamic of this molecule. Various direct and indirect approaches have already been utilized to detect NO level. NO can be measured by using electrochemical, hemoglobin, gas chromatography, mass spectrometry and chemiluminescence methods (Murphy and Noack, 1994; Dean and Harper, 1986; Wildt et al., 1997). The electrochemical method monitors the concentration NO directly and continuously (Malinski and Taha, 1992). It is based on the quantification of NO release in the presence of Cu^{+} /Cu^{2+} by amperometric measurement. Indirect methods rely on monitoring a molecular species or a physiological effect, which reflects the presence of NO. The chemiluminiscence is based on the reaction of NO with H_2O_2 to form peroxinitrite, and then this oxidizing species is quantified by reaction with luminal (Kikuchi et al., 1993; Malyshev et al., 1999). Hemoglobin has been reported to react with ROS including NO. The NO-

sensitive fluorescence dyes, 4,5-diaminofluorescin (DAF-2) and diaminorhodamine (DAR-4M) were developed (Kojima et al., 1998; Nakatsubo et al., 1998; Köjima et al., 2000 and 2001). These dyes do not fluorescence until they react with NO and thus the relative fluorescence of NO content can be monitored by using a fluorometer or a fluorescence microscope. DAF-2 DA does not react with ROS (Foissner et al., 2000). Conflicting data regarding the specificity of DAF-2 DA was reported that the reaction of DAF-2 DA with calcium increased the sensitivity in the presence of NO (Beligni et al., 2002). However, calcium alone had no effect on fluorescence formation (Broillet et al., 2001). Moreover, DAF-2 DA reacts with various NO donors in dose dependent manner, while superoxide, hydrogen peroxide, peroxynitrite or nitroxyl fail to change the fluorescence intensity of the probe (Roychowdhury et al., 2002). In this study, no detectable fluorescence was observed in the absence of NO sources such as plant cells and NO donors.

PTIO is known to be a specific NO scavenger which directly extinguishes NO generated by NO synthase (NOS) in animals without affecting NOS activity (Maeda et al., 1994). NO production by PAs was partially inhibited by PTIO (Begum, 2005). The competition between DAR-4M and PTIO to bind NO released from NO donor was observed in the cell-free medium showing the partial inhibition of fluorescence formation. More recently, Arita and Yamasaki (2005) reported that PTIO did not decrease the fluorescence formation by DAF-2, but rather increased it. Caution must be taken to use these chemicals in further NO detection experiments, until the most suitable method has been found.

4.2.3 Potential Role of NO and PAs in Abiotic Stress

Various abiotic stresses such as drought, low and high temperature, UV and ozone exposure induce the generation of ROS (Neill et al., 2002). ROS trigger various signaling pathways and also initiate several oxidatively destructive processes. Therefore it is necessary to maintain the appropriate low ROS levels for a survival response. In fact, NO reacting with ROS might serve as an antioxidant function during various stresses (Beligni and Lamattina, 1999a and 1999b). Modulation by NO on superoxides formation (Caro and Puntarulo, 1999) and inhibition of lipid peroxidation (Boveris et al., 2000) illustrate its potential role as an antioxidant. NO responses to

heat and chilling have been reported (Lamattina et al., 2003). NO production was increased in heat stressing alfalfa (Leshem, et al., 1998) and NO mediates chilling resistance (Lamattina et al., 2003). The high levels of ROS accumulation by heat and chilling stress might be suppressed by the antioxidant properties of NO and resulting in resistance to abiotic stress. The heat tolerant cotton tends to increase Spd and Spm pools at the expense of Put. In rice callus under heat stress, the levels of free and bound PAs are higher in the tolerant cultivar than in the non-tolerant one (Roy and Ghosh, 1996). Put accumulation at low temperature has been reported in many species. Several chilling tolerant plants, such as wheat, cherimoya, zucchini and rice, responded to low temperature with uniform and substantial increases in Spd and Spm. Spd and Spm may prevent chilling injury by a mechanism involving protection of membrane lipids through retarding lipid peroxidation or by their antioxidant properties (Flores, 1991). Salt stress and osmotic stress in rice and salt stress in sorghum, maize and tomato enhanced PA biosynthesis and can be ameliorated by PAs (Bouchereau et al., 1999). The osmotic stress and, partially, the salt stress mediating hormone abscisic acid also used NO as a mediator (Neill et al., 2003). Both NO- and PA-mediated stress responses might be linked by using NO as an intermediate.

Ozone, a major air pollutant, caused inhibition of photosynthesis, leaf injury and accelerates senescence. Exogenously supplied PAs suppress ozone-induced injury which is reported in tomato, tobacco, Norway spruce, wheat and potato. This protective role of PAs against ozone damage could be due to their free radical scavenging properties (Bouchereau et al., 1999). Ozone treatment of *Arabidopsis* plants induces NOS activity that precedes accumulation of salicylic acid (SA) and cell death (Rao and Davis, 2001). Since NO and PAs are involved in abiotic stress responses and NO is released by PAs as presented here, it is suggested that NO might play as a mediator in PA responses.

4.2.4 Plant Defence Responses and Potential Role of PAs and NO

A prominent common process between NO and PAs is their potential role in defence responses of plants to pathogen attack (Delledonne et al., 1998; Durner et al., 1998; Yamakawa et al., 1998; Foissner et al., 2000; Delledonne et al., 2001; Wendehenne et al., 2001; Mo and Pua, 2002; Takahashi et al., 2003; Lamotte et al., 2004; Takahashi

et al., 2004; Zeier et al., 2004). Hypersensitive response (HR) is a defence process activated in plants in response to pathogen attack. Associated with HR is an oxidative burst, in which greatly increased reactive oxygen species (ROS) generation, the programmed cell death, and the activation of signaling pathways driving the expression of various defence-related genes. HR results in localized plant cell death, which in turn limits nutrient availability and thus growth and spreading of the invading pathogen. Treatment of soybean cultures with avirulent (HR-inducing) but not virulent (HR-noninducing) *Pseudomonas syringae* PV *glycinea*, induced rapid NO biosynthesis (Delledonne et al., 1998). Infection of tobacco plants with HR-inducing varieties of tobacco mosaic virus induced NOS activity and it was inhibited by NOS inhibitors (Durner et al., 1998). During HR triggered by tobacco mosaic virus infection, tobacco plants produced high levels of Spm, which in turn activated a set of pathogenesis-related genes (Yamakawa et al., 1998). The levels of Put, Spd and Spm were increased in HR of barley to the powdery mildew fungus *Blumeria graminis.* Sp. hordeum (Cowley and Walters, 2002).

Spm stimulated the up-regulation of two important MAPKs of tobacco leaves involved in plant defence, wound-induced protein kinase and salicylic acid induced protein kinase, and defence genes expression were reported (Takahashi et al., 2003; Takahashi et al., 2004). Rapid regulation of NO by Spm as presented here has given a suggestion that NO might play a signaling intermediate for the Spm-activated MAP kinases upregulation.

4.2.5 Antisenescence and Potential Role of NO and PAs

Another notable common property between NO and PA is their antisenescence effects in plants. Ethylene plays an active role in senescence processes in plant. NO and ethylene have antagonistic effect in the senescence of plants. Exogenous application of NO extended the post harvest life of fresh horticultural products by inhibiting ethylene production (Leshem et al., 1998). Moreover, endogenous NO and ethylene content maintain an inverse correlation during the ripening of strawberries and avocados (Leshem and Pinchasov, 2000). While unripe, green fruits contain high NO and low ethylene concentrations, the maturation process is accompanied by a marked decrease of NO concomitant with an increase of ethylene (Leshem and Pinchasov, 2000).

Therefore, the role of NO in antisenescence could be the inhibition of ethylene formation, counteracting oxidative stress and scavenging free radicals. The anti-senescence effects of PAs are reported in rose (Sood and Nagar, 2003); *Vigna mungo* root nodules (Lahiri et al., 2004), *Arabidopsis* leaves (Woo et al., 2004), honey dew muskmelon fruit (Lester, 2000), oat (Bestford et al., 1993), and rice (Cheng et al., 1984; Bais and Ravishankar, 2002). It was suggested that this antisenescence effect might occur through retention of chlorophyll, inhibition of RNAse and protease activities, inhibition of ethylene synthesis, stabilization of membranes, prevention of lipid per oxidation and scavenging of free radicals.

Moreover, Spd and Spm were shown to reduce ethylene synthesis by inhibition of ACC synthase, which synthesizes ethylene. Spd and Spm were also effective in reducing chlorophyll breakdown in the dark in many cereal and dicot leaves, the order of activity being Spm> Spd (Bouchereau et al., 1999). It has been long known that cytokinin delays senescence and has the same property of retaining chlorophyll (Taiz and Zeiger, 1998). It had reported cytokinin-stimulated rapid NO biosynthesis (Tun et al., 2001) and rapid NO production induced by Spm in *Arabidopsis* here (Fig. 26). Taken together, there might be a link between NO, cytokinin and polyamine in the antisenescence of plants.

The physiological developmental processes in plants by PAs and NO have many similarities especially in pathogen defense, abiotic stress resistance and antisenescence responses. Although how NO and PAs are linked to each other is still not clear, It could be proposed that NO might be involved in these PA-induced development process as a mediator.

5 CONCLUSION

Recent years have seen a huge increase in research activities aimed at elucidating the biological roles of NO in plants and the underlying signaling mechanisms. The presented work here contributes on the role of NO in the signal transduction of the plant hormone cytokinin. Still our understanding of the signaling function of NO in cytokinin signaling is very limited. Although it was shown that NO induction by zeatin was dependent on the cytokinin receptor AHK4, the involvement of two more cytokinin receptors AHK2 and AHK3, in NO-cytokinin signaling remains to be clarified. However, one or more of the histidine phosphotransfer proteins, AHP1, AHP2 or AHP3, might be involved in NO-mediated cytokinin process as investigation of a triple knockout line suggested. It is necessary to confirm which AHP protein is connected to cytokinin-induced NO signaling and the role of two remaining AHPs proteins needs to be characterized. Although type-A response regulator *ARR5* failed to respond to the NO donors, the regulation of the expression patterns of the other 9 members of type-A *ARRs* by NO donors and NO inhibitors have not been studied yet. The role of type-B ARRs in NO-cytokinin signaling is still unknown.

Two important NO biosynthesis enzymes, NR and *At*NOS1, have been analyzed here but seem not to be linked to cytokinin-induced NO biosnythesis. NO biosynthetic enzymes activated by cytokinin still remain to be identified.

The important role of Spm and Spd in NO biosynthesis in plant NO are reported here. These presented work will open the door for a new excitating field of NO and polyamine research in future. There can be no doubt about the importance of NO in plant growth and development so plant biologists must go on to explore the biochemical functions of such a simple and fascinating molecule.

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7 APPENDIX

Appendix i. Modified Murashige & Skoog (MS) medium.

Macro elements stock solution (1l)

KH_2PO_4	1.7 g
$CaCl_2.2H_2O$	4.4 g
NH_4NO_3	16.5 g
$MgSO_4.7H_2O$	3.7 g
KNO_3	19.0 g

Micro elements stock solution (1l)

$MnSO_4.H_2O$	1.60 g
H_3BO_3	0.62 g
$ZnSO_4.7H_2O$	0.86 g
$Na_2MoO_4.2H_2O$	0.25 g
$CuSO_4.5H_2O$	0.02 g
$CoCl_2.5H_2O$	0.002 g
KI	0.008 g

Fe-EDTA stock soultion (1l)

$FeSO_4.H_2O$	1.4 g
Na_2EDTA	1.9 g

Vitamins stock soultion (1l)

Nicotic acid	0.1 g
Thiamin HCl	1.0 g
Pyrodoxol HCl	0.1 g
Myo-inositol	10.0 g

MS medium was prepared as follows (1l)

Macro element stock solution	100 ml
Micro element stock solution	10 ml
Fe-EDTA stock solution	20 ml
Vitamin	10 ml
Glucose	30.0 g
Glycine	0.02 g

pH 5.7 adjusted before autoclave with KOH.

Phytoagar 12 g/l for solid MS

The medium was autoclaved for 15 minutes at 121^{o}C. After autoclave required amount of

hormone or polyamines was added.

Half strength of MS (1l)

Macro element stock solution	50 ml
Micro element stock solution	5 ml
FE-EDTA stock solution	10 ml
Vitamin	5 ml
Glucose	15.0 g
Glycine	0.01 g

pH 5.7 adjusted before autoclave with KOH.

No phytoagar was added in liquid medium. The medium was autoclaved for 15 minutes at 121°C.

Appendix ii. Sterilisation solution

Sodium hypochlorite	4 %
Triton X-100	0, 2%

Arabidopsis Seeds were Sterilized as follow:

Twenty milligrams (mg) of the *Arabidopsis* seeds were weighted and transferred into the 2 ml sterilized eppendorf tube. One (millilitre) ml of 70% ethanol was added to the tube and shaken gently for 10 minutes. Then ethanol was removed. One ml of sterilizing solution (3 ml of distilled water + 3 ml of 12% sodium hypochlorite + 2 µl of Triton X) was added and the tube was shaken gently for 10 minutes. The solution was taken away and the seeds were rinsed 5 to 7 times with double distilled water. The seeds were kept in double distilled water at +4°C over night. The next day, the water was removed and the seeds were washed once with double distilled water.

Appendix iii. Hormones and PAs preparations

Zeatin solution (1 mg/ml)

Dissolve 2.192 mg of zeatin in 2.5 ml of (1N) NaOH (Sodium hydroxide) and then in 7.5 ml H_2O for 100 mM stock. 100 mM zeatin was dissolved in 1 N NaOH and further with diluted double distilled water. The same amount of NaOH in every sample was added to avoid the side effect of this chemical on NO production] and with or without PTIO or AET [dissolved in distilled water.

Spermine (50 mM)

101.17 mg dissolved in 2.5 ml of H_2O and then in 7. 5 ml 30 mM MES buffer.

Spermidine (50 mM)

127.3 mg dissolved in 2.5 ml of H_2O and then in 7.5 ml 30 mM MES buffer.

Putrescine (50 mM)

80.54 mg dissolved in 2.5 ml of H_2O and then in 7.5 ml 30 mM MES buffer.

Arginine (50 mM)

43.55 mg dissolved in 2.5 ml of H_2O and then in 7.5 ml 30 mM MES buffer.

All the polyamines and Arg stock soultion were adjusted to pH 5.7 with hydrochlorid acid.

Appendix iv. 50 mM Phosphate buffer

It was prepared with 8.709 g of K_2HPO_4 and 6.8055 g of KH_2PO_4 in 1 liter of distilled water. The pH was adjusted to pH 7.4 by mixing them.

Appendix v. 30 mM MES monohydrate buffer

MES (6.3975 g) was dissolved in one litre of distilled water and pH was adjusted to 5.7.

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Publications

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