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NO way!

Is nitric oxide level in tomato regulated by a mammalian IKK/NFκB-like signaling pathway?

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Nitric oxide (NO) is an essential signaling molecule in plants. However little is known about signaling pathways regulating NO levels in plants. Recently we reported a NO overproducing mutant of tomato that had extremely short roots (*shr*) at seedling stage. The scavenging of NO restored root elongation in the *shr* mutant providing us with a convenient bioassay to analyze the signaling pathway upstream of NO production. The application of previously reported pharmacological inhibitors of ubiquitin-proteasome signaling caused a drastic reduction in NO levels and restored root elongation in the mutant. Since these pharmacological inhibitors specifically inhibit mammalian IKK/NFκB signaling, we propose that a pathway functionally similar to IKK/NFκB pathway regulates NO levels in tomato.

Key words: nitric oxide, root growth, short root mutant (*shr*), pharmacological inhibitors, NFκB, tomato (*Solanum lycopersicum*)

Abbreviations: *shr,* short root mutant; NO, nitric oxide; NOS, nitric oxide synthase

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Nitric oxide (NO) is a bioactive gaseous molecule that functions as an essential signaling molecule in plants.[1-3] NO has been shown to be involved in the signaling of growth, development and adaptive responses to multiple stresses in plants.[4-9] In plants, NO is assumed to be synthesized by several mechanisms of which two distinct pathways are a nitrite pathway likely via nitrate reductase[10] and an arginine pathway via a nitric oxide synthase (NOS) like enzyme.[2,11-13] Several plant hormones and light appear to regulate NO levels in plants.[1] NO brings about its biological functions through its action on multiple downstream signaling pathways that includes generation of cGMP, cADPR and elevation of cytosolic calcium.[1] Though diverse responses regulate NO formation,[1] little is known about the molecular mechanisms regulating production of NO in plants. In mammals, NO is produced by action of three different NOS, namely constitutively expressed endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS).[14,15] The induction of iNOS is considered to be regulated by several mechanisms including transcriptional regulation by the IκB kinase (IKK)/nuclear factor κB (NFκB) pathway.[16-19] NFκB is an inducible transcription factor that regulates the expression of a variety of genes such as adhesion factors, chemokines and cytokines, in addition to inducible enzymes such as iNOS. The studies on mechanism of NFκB activation have indicated that NFκB is sequestered in the cytoplasm of cells by the inhibitory IκB proteins. In response to a variety of stimuli and developmental responses, IκB is rapidly phosphorylated, ubiquitinated and degraded via proteasome, releasing NFκB for translocation into the nucleus to initiate transcription of genes such as of iNOS. IKK is the convergence point in most signaling pathways activated by many stimuli leading to the phosphorylation and degradation of IκB.

NO being a vital molecule for plant development, it is possible that its cellular level is regulated by a pathway similar to mammalian IKK/NFκB signaling pathway, which possibly regulates biosynthesis of NOS like enzyme(s). It has been shown

that Arabidopsis NIM1/NPR1 protein and AKR2 protein regulating pathogenesis related-1 (*PR*-1) gene expression involved in systemic acquired resistance (SAR) in plants have high homology to mammalian IB-like proteins,[20,21] and both of these proteins exert their regulatory function through interaction with other proteins. Though NFκB-like proteins have not been reported from plants, gene annotation studies have revealed that glutathione peroxidase,[22] phenylalanine-ammonia lyase (*PAL*),[23] and *PR-1*,[24] genes have motifs in their promoter elements that are analogous to NFκB-like protein binding sites. However, at the moment there is no conclusive evidence available of the existence of a signaling pathway similar to that established for IKK/NFκB in animal systems that regulates NO level in plants.

Enhanced NO Levels Causes Shortening of Root in the *shr* Mutant Seedlings

To elucidate the pathway regulating NO formation in plants we analyzed a tomato mutant that had extremely short roots (*shr*) at the seedling stage. Mutant plants showed elevated NO levels in all organs compared to the wild type, with significantly higher NO levels at the root tip.[25] Higher NO level in the *shr* mutant was also associated with increase in NOS activity in all organs. Though the *shr* mutant showed reduction in overall growth of adult plants, the most striking effect was the extreme reduction of primary root length of seedlings. Fascinatingly, application of NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) that is known to reduce endogenous NO levels, as well as NG-nitro-L-argininemethyl ester (L-NAME) an inhibitor of NOS activity stimulated root elongation along with significant decrease in NO levels in the *shr* root tips. These results provided compelling evidence that short root phenotype of the mutant seedlings is related to the overproduction of NO and restoration of root elongation can serve as an elegant bioassay to decipher the mechanism(s) regulating NO level in plants.

Inhibitors of Mammalian IKK/NFκB Pathway Rescues *shr* Seedling Phenotype

Presently the molecular nature of the components regulating NO level in plants remains to be determined. Although there is no priori knowledge about pathway regulating NO synthesis in plants, it is reasonable to assume that the signaling pathway regulating NO synthesis in plants may perhaps have some commonality to that observed in mammalian system. For example in animals, the signaling function of NO is mediated via a pathway involving soluble guanylyl cyclases that generate cGMP and phosphodiesterases that hydrolyze cGMP.[26] Though sequences homologous to mammalian guanylyl cyclases and phosphodiesterases have not been reported in plants, results obtained with use of pharmacological inhibitors provide evidence for cGMP function in NO-triggered signaling pathway[27] such as pollen tube growth[28] and root development.[29] The reduction in endogenous NO levels coupled with stimulation of root elongation in the *shr* mutant seedlings upon application of pharmacological inhibitors provides an ideal system for further elucidating the pathway(s) regulating NO synthesis in plants.

In mammals NO levels are regulated by IKK/NFκB signal transduction pathway via NFκB activation, a transcription factor associated with increased iNOS expression. We examined the likely operation of this pathway by monitoring the rescue of *shr* root elongation[25] using a series of pharmacological inhibitors specifically blocking this pathway.[30] In fact in plants, the existence of NOS like enzymic activity was first proposed based on reduction of NO levels on application of mammalian NOS inhibitors.[1] Therefore we reasoned that upstream signal transduction components regulating NO levels in plants might also show similar sensitivity to pharmacological inhibitors used in mammalian systems. Our studies revealed that the shortening of the root in the *shr* mutant can be reversed by application of inhibitors that can cause a reduction in endogenous NO levels.

NFκB, a family of transcriptional factors, is the key partner in the IKK/NFκB signaling pathway of mammalian system. A range of inhibitors is known that blocks different aspects of the action of NFκB.[30] One of the potent inhibitor of NFκB activity is glucocorticoid dexamethasone that induces increase in levels of IκBα resulting in reduced amounts of NFκB that translocates to the nucleus, thus inhibiting iNOS gene transcription and promoter activation by decreasing NFκB DNA binding activity.[31] Pyrrolidine dithiocarbamate (PDTC) is another widely used NFκB inhibitor that acts by hampering NFκB binding to DNA.[30] The reported stimulation of the *shr* root elongation by two diverse inhibitors of NFκB action suggests the existence of molecules with similar properties and affinity to these inhibitors in plant systems.

It is known that IKK phosphorylates mammalian IκBα proteins, and subsequently phosphorylated IκBα protein is targeted for polyubiquitination and subsequent degradation by the proteasome, thereby releasing NFκB. Application of a IKK-2 inhibitor V that selectively blocks IκBα phosphorylation and thereby prevents the induction of NFκB nuclear translocation,[30] also stimulated root elongation in *shr* seedlings. Similar results were also obtained with application of RO106-9920, a cell-permeable highly selective irreversible inhibitor of IκBα ubiquitination that blocks IκB degradation and NFκB activation.[32] It is known that peptide aldehyde N-carbobenzoxyl-L-leucinyl-L-leucinyl-L-norleucinal (MG-132) acts as a specific inhibitor of proteasome blocking IκBα degradation and NFκB activation.[30] As expected use of proteasome inhibitor MG-132 stimulated *shr* root elongation. In essence, the amelioration of root phenotype by above inhibitors point toward occurrence of a signaling pathway in plants, which perhaps operates in a fashion similar to IKK/NFκB signaling pathways of mammals.

A Mammalian IKK/NFκB-Like Signaling Pathway Regulates NO Level in Tomato

Bioassays provide a powerful method to predict and identify the components of several hormonal signaling pathways in plants. Additionally, phenocopying of

a mutant phenotype as well as rescue of the phenotype by application of bioactive molecules have been often used for inferring signaling pathways regulating such phenotypes. Ostensibly, stimulation of *shr* root elongation by pharmacological inhibitors specific for mammalian IKK/NFκB signaling pathway compellingly indicates a likely occurrence of similar pathway(s) in plants. It has been shown that Arabidopsis NIM1/NPR1 protein[20] and AKR2 protein regulating pathogenesis related-1 (*PR*-1),[21] gene expression have high homology to mammalian IκB-like proteins, and both of these proteins exert their regulatory function through interaction with other proteins. Though NFκB-like proteins have been not reported from plants, the gene annotation studies have revealed that phenylalanine-ammonia lyase (*PAL*),[23] and *PR*-1,[24] genes have in their promoter elements motifs that are analogous to NFκB-like protein binding sites. Mustard NPR1 protein when expressed in mammalian cell lines bound to NFκB and inhibited its nuclear translocation including downregulation of iNOS.[33] Based on the inhibitor data and upregulation of NO level, it appears that *shr* mutation affects a component upstream of NOS like enzyme. **Figure 1** shows a simple model that is consistent with our data[25] outlining a putative signaling pathway regulating NO levels in tomato seedlings. Since *shr* mutant shows upregulation of NO, the mutated gene product is probably a negative regulator, which in its native wild type form restrains NO production in plants. Based on our results we speculate that loss of this regulator either affects a component upstream of IKK/NFκB pathway, or perhaps decreases levels of a protein functionally similar to IκBα thus releasing a NFκB-like effector. In mammalian system where extensive knowledge has accumulated about NO synthesis, several mechanisms have been described that lead to upregulation of NOS activity. For example, human diseases such as asthma is believed to be caused by mutations in genes involved in the NFκB signaling pathway leading to constitutive NFκB activation and iNOS upregulation causing NO accumulation.[34] It is plausible that in *shr* mutant activation of a signaling component functionally similar to mammalian NFκB may be involved in upregulation of NOS activity and NO levels. In summary, we propose that operation of a IKK/NFκB-like signaling pathway regulates NO production in plants. Molecular identification of target proteins constituting this signaling pathway will be the next challenge in future.

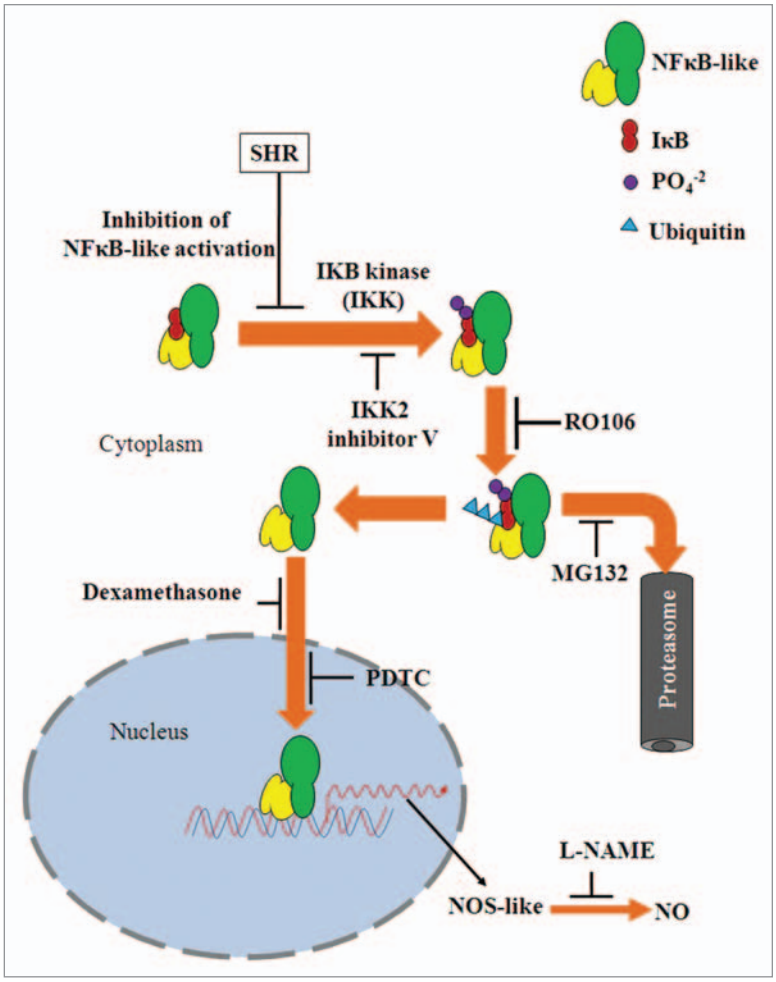

Figure 1. Schematic representation of IKK/NFκB-like signaling pathway regulating NO levels in tomato seedlings. The sites of action of the pharmacological inhibitors as derived from existing mammalian models are indicated. The putative role of native SHR as a repressor of a NFκB-like molecule is also indicated. The mutation in *shr* presumably results in the removal of this negative regulator thereby resulting in the constitutive transcriptional activation of a NOS-like enzyme. The increase in NOS-like activity leads to increase in NO levels in the mutant resulting in inhibition of root elongation.

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