Running Head: Ca\textsuperscript{2+}, CNGC, NO and leaf senescence in Arabidopsis

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Leaf senescence signaling: The Ca\(^{2+}\)-conducting Arabidopsis cyclic nucleotide gated channel2 acts through nitric oxide to repress senescence programming

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Ca^{2+} and nitric oxide (NO) are essential components involved in plant senescence signaling cascades. In other signaling pathways, NO generation can be dependent on cytosolic Ca^{2+}. The Arabidopsis (Arabidopsis thaliana) mutant dnd1 lacks a plasma membrane-localized cation channel (CNGC2). We recently demonstrated that this channel affects plant response to pathogens through a signaling cascade involving Ca^{2+} modulation of NO generation; the pathogen response phenotype of dnd1 can be complemented by application of an NO donor. At present, the interrelationship between Ca^{2+} and NO generation in plant cells during leaf senescence remains unclear. Here, we use dnd1 plants to present genetic evidence consistent with the hypothesis that Ca^{2+} uptake and NO production play pivotal roles in plant leaf senescence. Leaf Ca^{2+} accumulation is reduced in dnd1 leaves compared to wild type. Early senescence-associated phenotypes (such as loss of chlorophyll, expression level of senescence associated genes, H_{2}O_{2} generation, lipid peroxidation, tissue necrosis, and salicylic acid levels) were more prominent in dnd1 leaves compared to wild type. Application of a Ca^{2+} channel blocker hastened senescence of detached wild type leaves maintained in the dark; increasing the rate of chlorophyll loss, expression of a senescence associated gene, and lipid peroxidation. Pharmacological manipulation of Ca^{2+} signaling provides evidence consistent with genetic studies of the relationship between Ca^{2+} signaling and senescence with the dnd1 mutant. Basal levels of NO in dnd1 leaf tissue were lower than that in leaves of wild type plants. Application of an NO donor effectively rescues many dnd1 senescence related phenotypes. Our work demonstrates that the CNGC2 channel is involved in Ca^{2+} uptake during plant development beyond its role in pathogen defense response signaling. Work presented here suggests that this function of CNGC2 may impact downstream 'basal' NO production in addition to its role (also linked to NO signaling) in pathogen defense responses, and that this NO generation acts as a negative regulator during plant leaf senescence signaling.
INTRODUCTION

Senescence can be considered as the final stage of a plant’s development. During this process, nutrients will be reallocated from older to younger parts of the plant such as developing leaves and seeds. Leaf senescence has been characterized as a type of programmed cell death (PCD) (Lim et al., 2003; Quirino et al., 2000; Gan and Amasino, 1997). During senescence, organelles such as chloroplasts will break down first. Biochemical changes will also occur in the peroxisome during this process. When the chloroplast disassembles, it is easily observed as a loss of chlorophyll. Mitochondria, the source of energy for cells, will be the last cell organelles to undergo changes during the senescence process (Quirino et al., 2000). At the same time, other catabolic events (e.g. protein and lipid breakdown etc.) are occurring (Quirino et al., 2000). Hormones may also contribute to this process (Gepstein, 2004). From this information we can infer that leaf senescence is regulated by many signals.

Darkness treatment can induce senescence in detached leaves (Ülker et al., 2007; Guo and Crawford, 2005; Chrost et al., 2004; Weaver and Amasino, 2001; Chou and Kao, 1992; Poovaiah and Leopold, 1973). Ca^{2+} can delay the senescence of detached leaves (Poovaiah and Leopold, 1973) and leaf senescence induced by methyl jasmonate (MeJA) (Chou and Kao, 1992); the molecular events that mediate this effect of Ca^{2+} are not well characterized at present.

Nitric oxide (NO) is a critical signaling molecule involved in many plant physiological processes. Recently published evidence supports NO acting as a negative regulator during leaf senescence (Mishina et al., 2007; Guo and Crawford, 2005). Abolishing NO generation in either loss of function mutants (Guo and Crawford, 2005) or transgenic Arabidopsis (Arabidopsis thaliana) plants expressing NO degrading dioxygenase (NOD) (Mishina et al., 2007) leads to an early senescence phenotype in these plants as compared to wild type (WT). Corpas et al., (2004) showed that endogenous NO is mainly accumulated in vascular tissues of pea (Pisum sativum) leaves. This accumulation is significantly reduced in senescing leaves (Corpas et al., 2004). Corpas et al., (2004) also provided evidence that NO synthase (NOS)-like activity (i.e., generation of NO from L-arginine) is greatly reduced in senescing leaves. Plant NOS
activity is regulated by Ca\(^{2+}\)/calmodulin (CaM) (Corpas et al., 2009, 2004; Ma et al., 2008; Valderrama et al., 2007; del Río et al., 2004; Delledonne et al., 1998). These studies suggest a link between Ca\(^{2+}\) and NO that could be operating during senescence.

In animal cells, all three NOS isoforms require Ca\(^{2+}\)/CaM as a cofactor (Alderton et al., 2001; Stuehr, 1999; Nathan and Xie, 1994). Notably, animal NOS contains a CaM binding domain (Stuehr, 1999). It is unclear whether Ca\(^{2+}\)/CaM can directly modulate plant NOS, or if Ca\(^{2+}\)/CaM impacts plant leaf development/senescence through (either direct or indirect) effects on NO generation. However, recent studies from our lab suggest that Ca\(^{2+}\)/CaM acts as an activator of NOS activity in plant innate immune response signaling (Ma et al., 2008; Ali et al., 2007). Although Arabidopsis NO ASSOCIATED PROTEIN1 (AtNOA1; formerly named AtNOS1) was thought to encode a NOS enzyme, no NOS-encoding gene has yet been identified in plants (Crawford et al., 2006; Zemojtel et al., 2006; Guo et al., 2003). However, the AtNOA1 loss-of-function mutant does display reduced levels of NO generation and several groups have used the NO donor sodium nitroprusside (SNP) to reverse some low-NO related phenotypes in Atnoa1 plants (Zhao et al., 2007; Bright et al., 2006; Guo et al., 2003). Importantly, plant endogenous NO-deficiency- (Mishina et al., 2007; Guo and Crawford, 2005) or abscisic acid- (ABA)/MeJA (Hung and Kao, 2004, 2003) induced early senescence can be successfully rescued by application of exogenous NO. Addition of NO donor can delay gibberellin (GA)-elicited PCD in barley (Hordeum vulgare) aleurone layers as well (Beligni et al., 2002).

It has been suggested that salicylic acid (SA), a critical pathogen defense metabolite, can be increased in natural (Mishina et al., 2007; Morris et al., 2000) and transgenic NOD induced senescent Arabidopsis leaves (Mishina et al., 2007). Pathogenesis related gene 1 (PR1) expression is upregulated in transgenic Arabidopsis expressing NOD (Mishina et al., 2007) and in leaves of an early senescence mutant (Ülker, et al., 2007). Plant cyclic nucleotide gated channels (CNGCs) have been proposed as candidates to conduct extracellular Ca\(^{2+}\) into the cytosol (Ma et al., 2009a, 2009b; Ali et al., 2007; Demidchik and Maathuis, 2007; Kaplan et al., 2007; Frietsch et al., 2007; Ma and Berkowitz, 2007; Urquhart et al., 2007; Lemtiri-Chlieh and Berkowitz, 2004; Talke
et al., 2003; Sunkar et al., 2000). Arabidopsis ‘defense, no death’ (dnd1) mutant plants have a null mutation in the gene encoding the plasma membrane localized Ca\(^{2+}\)-conducting CNGC2 channel. This mutant also displays no hypersensitive response to infection by some pathogens (Ali et al., 2007; Clough et al., 2000). In addition to involvement in pathogen mediated Ca\(^{2+}\) signaling, CNGC2 has been suggested to participate in the process of leaf development/senescence (Köhler et al., 2001). dnd1 mutant plants have high levels of SA and expression of PRI (Yu et al., 1998) and spontaneous necrotic lesions appear conditionally in dnd1 leaves (Jirage et al., 2001; Clough et al., 2000). Endogenous H\(_2\)O\(_2\) levels in dnd1 mutants are increased from WT levels (Mateo et al., 2006). Reactive oxygen species molecules such as H\(_2\)O\(_2\) are critical to the PCD/senescence processes of plants (Zimmermann et al., 2006; Guo and Crawford, 2005; Hung and Kao, 2004; Navabpour et al., 2003; Overmyer et al., 2003). Here, we use the dnd1 mutant to evaluate the relationship between leaf Ca\(^{2+}\) uptake during plant growth and leaf senescence. Our results identify NO, as affected by leaf Ca\(^{2+}\) level, to be an important negative regulator of leaf senescence initiation. Ca\(^{2+}\)-mediated NO production during leaf development could control senescence associated gene (SAG) expression as well as the production of molecules (such as SA and H\(_2\)O\(_2\)) that act as signals during the initiation of leaf senescence programs.
RESULTS

Leaf Ca\(^{2+}\) accumulation is reduced in the dnd1 mutant

Ca\(^{2+}\) conducting ion channels facilitate transitory cytosolic Ca\(^{2+}\) ‘spikes’ as components of numerous signaling pathways in plant cells. They are also thought to play a role in Ca\(^{2+}\) nutrition; i.e., uptake and translocation of Ca\(^{2+}\) within the plant (Hetherington and Brownlee, 2004; White and Broadley, 2003). Patch clamp studies of plant cells also indicate that nonselective, weakly voltage gated (i.e. potentially ligand gated) channels contribute to Ca\(^{2+}\) uptake in plants (Demidchik and Maathuis, 2007; White and Broadley, 2003; White et al., 2002). However, current reviews indicate no gene product has yet been associated with Ca\(^{2+}\) uptake into plants or accumulation in leaves (Maathuis, 2009). Therefore, it is currently unclear from prior work which cation channels contribute to Ca\(^{2+}\) uptake into plants and accumulation in leaves during plant growth and development (in contrast to temporary influx of Ca\(^{2+}\) associated with signaling).

Of the 57 known cation conducting channels in Arabidopsis, 20 members are CNGCs. CNGCs are candidates for specific gene products involved in the plant Ca\(^{2+}\) uptake pathway (Demidchik and Maathuis, 2007; White and Broadley, 2003; White et al., 2002). The cytosolic secondary messenger cAMP activates inward Ca\(^{2+}\) current through the plasma membrane in WT leaf cells while this current is absent in leaves of the CNGC2 loss-of-function mutant dnd1 (Ali et al., 2007; Lemtiri-Chlieh and Berkowitz, 2004). Pathogen recognition results in activation of CNGC-dependent inward Ca\(^{2+}\) current and downstream NO production during plant innate immune response signaling cascades (Ma et al., 2009a; Ali et al., 2007). These studies link CNGC channels with inward Ca\(^{2+}\) flux associated with signaling.

Here, we first investigated whether CNGC2 plays a role in Ca\(^{2+}\) uptake from a plant nutrition perspective by comparing leaf Ca\(^{2+}\) content in WT and dnd1 plants grown with different Ca\(^{2+}\) levels in their growth medium. We found that the CNGC2 null mutation in dnd1 plants has effects on long-term Ca\(^{2+}\) acquisition (Figure 1) at a range of external Ca\(^{2+}\), either when we measured total shoot Ca\(^{2+}\) levels in plants grown on solid
(agar) medium (experiments 1 and 2), or grown hydroponically on liquid nutrient solution (experiment 3 and 4). In these experiments (Figure 1), we observed a decrease in Ca$^{2+}$ content of leaves of dnd1 plants as compared to WT plants under a number of different growth conditions (solid and liquid media, and varying external Ca$^{2+}$). These results are consistent with a hypothesis that conductance through cation channels formed by CNGC2 contributes to Ca$^{2+}$ nutrition of the plant. It appears that CNGC2 channels contribute to Ca$^{2+}$ acquisition by leaves as part of the normal growth and development of the plant in addition to the Ca$^{2+}$ conductance associated with innate immune signaling described by Ali et al. (2007) and Ma et al. (2009a).

The work shown in Figure 1 is presented because the results are consistent with function of CNGC2 as a Ca$^{2+}$ uptake pathway in leaves during growth and development (i.e. in addition to its role in immune responses). We do not assert here that the levels of leaf Ca$^{2+}$ found in WT and dnd1 plants as shown in Figure 1 are specifically causal to differences in senescence programming in WT and mutant plants. Rather, we speculated that since CNGC2 is functioning as a Ca$^{2+}$ uptake pathway during normal growth of WT plants, this channel may be responding to as-yet-unidentified signals during growth that impact the onset of senescence in leaves. CNGC2 loss-of-function in dnd1 plants could impact this signaling.

CNGC2 is involved in plant leaf development/senescence signaling; the CNGC2 loss-of-function mutant (dnd1) displays early senescence phenotypes

Results (Figure 1) indicating that CNGC2 provides a pathway for Ca$^{2+}$ uptake in plants beyond that related to immune signaling led us to investigate the link between CNGC2 and leaf senescence. We observed that dnd1 plants show early leaf senescence phenotypes compared to WT plants (Figure 2). In Figure 2A, senescent yellowing leaves can be observed on dnd1 plants (highlighted by arrows) of the same age as WT plants, which do not have any leaves undergoing senescence at this point in plant development. The sporadic development of leaf tip yellowing shown here for dnd1 plants appears similar to that shown by Mishina et al. (2007) for plants with (apparently) low basal NO levels. Mishina et al. found that about a week after expression of an NOD, Arabidopsis
plants had similar level and extent of leaf yellowing as shown here for dnd1 plants; this developmental change corresponds to senescence stage 'S2' (Mishina et al., 2007).

Previous studies have reported that spontaneous necrotic lesions can appear in dnd1 leaves (Jirage et al., 2001; Clough et al., 2000). Here, our observation further expands our understanding of the dnd1 mutant phenotype. Subjecting detached young leaves to darkness also revealed that dnd1 leaves achieve senescence faster than WT leaves (Figure 2B and C). With leaves from WT plants, Gd$^{3+}$, a Ca$^{2+}$ channel blocker, hastens senescence, mimicking the early senescence phenotype of dnd1 leaves (Figure 2B and C). Results in Figure 2A-C are consistent with the hypothesis that the presence of a functional Ca$^{2+}$ uptake pathway may act to defer senescence during development.

A recent microarray study from Chan et al. (2008) provides a wealth of information about differences in gene expression associated with the CNGC2 loss-of-function mutant. Among the genes whose expression were upregulated in CNGC2 loss-of-function mutants as suggested by the global analysis undertaken by Chan et al. (2008), some are intimately associated with the senescence process (for information about those gene expression profiles in Arabidopsis tissues refer to Arabidopsis eFP Browser at bar.utoronto.ca, Winter et al., 2007). Here, we monitored the expression of SAGs (WRKY70, RLK5 and At5g10760) in dnd1 and WT plants using the semi-quantitative reverse transcriptional polymerase chain reaction (RT-PCR); demonstrating upregulation of these SAGs in leaves of dnd1 as compared to WT plants (Figure 2D). We also found increased PRI transcript levels in dnd1 plants (Figure 2D), similar to what was reported by Yu et al. (1998). Furthermore, measurements of lipid peroxidation (i.e., quantified by monitoring malondialdehyde (MDA) levels), which increases during senescence (Wingler et al., 2004; Berger et al., 2001; Buchanan-Wollaston, 1997; Dhindsa et al., 1981) indicate that dnd1 leaves have a higher level of lipid peroxidation than WT leaves (Figure 2E). A number of different experimental approaches as delineated in Figure 2, then, indicate that senescence 'programs' are activated in leaves of dnd1 plants as compared to leaves of WT plants.

Results in Figure 2B and C indicate that with regard to the rapid senescence that occurs in detached leaves kept in the dark, application of Gd$^{3+}$, a channel blocker that prevents inward currents through plasma membrane Ca$^{2+}$ conducting channels (Tegg et
al., 2005), mimics the phenotype shown by \textit{dnd1}. Further studies were undertaken to determine if the effect of Gd\textsuperscript{3+} on senescence programming could be observed at the biochemical and gene expression level. Expression of \textit{SAG12} is increased during senescence of detached leaves kept in the dark, as well as in planta in leaves undergoing natural senescence; not all SAG genes upregulated in planta show a similar response to darkness in detached leaves (Weaver et al. 1998). Quantitative real-time PCR (qPCR) evaluation of \textit{SAG12} expression in detached WT leaves kept in the dark demonstrated that the hastening of senescence by Gd\textsuperscript{3+} could also be observed at the level of gene expression (Figure 2F). \textit{SAG12} expression was greater at days 1-3 after detachment (Figure 2F) in WT leaves when they were exposed to Gd\textsuperscript{3+}. Further studies indicated that hastening of senescence by Gd\textsuperscript{3+} could be observed at the biochemical level as well. With detached leaves kept in the dark, MDA level increased more quickly in the presence of Gd\textsuperscript{3+} (Supplemental Figure 1). In other experiments, \textit{SAG12} expression was compared in leaves of WT and \textit{dnd1} plants (Supplemental Figure 2). \textit{SAG12} expression was noted in leaves of \textit{dnd1} plants and not in leaves of WT plants (Supplemental Figure 2, day ‘0’). When leaves were detached from plants and kept in the dark, further \textit{SAG12} expression occurred in \textit{dnd1} leaves earlier than in WT leaves (compare expression on day 1 in Supplemental Figure 2). Results from all of these aforementioned studies (Figure 2, Supplemental Figure 1, and Supplemental Figure 2) indicate that impairment of a leaf cell Ca\textsuperscript{2+} uptake pathway (either genetically or biochemically) hastens senescence programming.

\textit{dnd1} has a lower endogenous NO level in leaves compared to WT plant

The hypothesis underlying the work presented in this report is that impairment of a Ca\textsuperscript{2+} uptake pathway functional during growth and development of \textit{dnd1} plants leads to loss of signal that represses senescence programming in leaves. We speculate that CNGC2-dependent Ca\textsuperscript{2+} uptake affects leaf senescence programming through an intermediary step of NO synthesis. Thus, in \textit{dnd1} plants with a null mutation in CNGC2, downstream Ca\textsuperscript{2+}-dependent activation of NO generation may be impaired. Reduced NO generation could lead to a derepression of leaf senescence programming. The basis for
this conjecture is as follows. A) Prior studies (Mishina et al., 2007; Guo and Crawford, 2005; Corpas et al., 2004; Chou and Kao, 1992; Poovaiah and Leopold, 1973) discussed above suggest that both leaf Ca\(^{2+}\) and NO repress leaf senescence. B) High levels of SA and expression of \(PRI\) are associated with treatments that reduce NO production in leaves and induce early senescence (Mishina et al., 2007; Ülker, et al., 2007; Morris et al., 2000); SA level and \(PRI\) expression are elevated in leaves of \(dnd1\) plants (Yu et al., 1998). C) Senescing leaves have reduced endogenous NO level (Corpas et al., 2004). D) Recent studies from this lab (Ali et al., 2007) indicate that impairment of innate immune signaling in \(dnd1\) plants occurs due to a lack of Ca\(^{2+}\)-dependent NO generation. We measured the endogenous NO level in WT and \(dnd1\) leaves. As shown in Figure 3, \(dnd1\) plants have lower endogenous levels of leaf NO as compared to WT.

Endogenous basal NO (in contrast to NO generation in response to a specific signal) in leaves of any Arabidopsis genotype has not been previously reported. In prior studies from this lab (Ali et al., 2007), basal level of NO in guard cells was monitored in epidermal peels of WT and \(dnd1\) plant leaves. No significant differences were noted although the level in \(dnd1\) guard cells was slightly higher. The difference between these prior results focusing on guard cells and the work reported here may be due to the measurement of total leaf NO in the work shown in Figure 3.

**Nitric oxide donor application rescues \(dnd1\) senescence associated phenotypes**

With regard to impaired pathogen response signaling in \(dnd1\) plants, application of the NO donor SNP to these mutant plants complemented the \(dnd1\) pathogen response phenotype (Ali et al., 2007). We therefore tested the model that is the focus of the work presented here about involvement of NO in Ca\(^{2+}\) repression of senescence programming by examining the effect of SNP (an NO donor) application on some senescence-related phenotypes in leaves of \(dnd1\) plants. As is the case with other NO donors used with plants, NO release from SNP requires light (Floryszak-Wieczorek et al., 2006). Therefore, our evaluation of SNP effects on senescence in \(dnd1\) plants focused on phenotypes occurring in the light.
As mentioned above, prior studies have shown that \textit{PRI} expression and SA levels are elevated in \textit{dnd1} plants as compared to WT plants (Yu et al., 1998). We confirmed these results (results not shown, also see Figure 2D). We also find that SNP treatment reduced the high constitutive \textit{PRI} transcriptional level in \textit{dnd1} (Figure 4A). In addition, SNP application to \textit{dnd1} plants also (modestly) lowered the constitutively high level of SA accumulation (Figure 4B). SNP application had no significant effect on either \textit{PRI} expression or SA levels in leaves of WT plants (results not shown).

Effect of exogenous NO application was further tested on other senescence related phenotypes in \textit{dnd1} plants. Increased endogenous H\textsubscript{2}O\textsubscript{2} content (reported by Mateo et al., 2006) and extent of necrotic regions in leaves (reported by Jirage et al., 2001) of \textit{dnd1} plants decreased after the addition of SNP (Figure 5A and B respectively). Although only qualitative effects of SNP treatment can be discerned from the images shown in Figures 5A and 5B, it appears that the effect of exogenous NO supply on \textit{dnd1} plants, i.e. reduced H\textsubscript{2}O\textsubscript{2} and necrosis, is not induced by SNP in leaves of WT plants. SNP application also was found to have an effect on the high level of lipid peroxidation in \textit{dnd1} plants (Figure 2E). As shown in Figure 5C, application of the NO donor SNP reduced the high level of lipid peroxidation in \textit{dnd1} leaves while SNP had no effect on the level of lipid peroxidation in leaves of WT plants.

The expression level of the SAGs \textit{At5g10760}, \textit{WRKY70}, \textit{RLK5} and \textit{PRI} is increased in (attached) leaves of \textit{dnd1} plants (Figure 2D). Application of the NO donor SNP to \textit{dnd1} plants reduced the expression level (monitored using RT-PCR) of these SAGs (Figure 5D). Analysis using qPCR also demonstrated that exogenous NO application reduced expression of \textit{PRI} in leaves of \textit{dnd1} plants; SNP had no significant effect on \textit{PRI} expression in leaves of WT plants (Supplemental Figure 3). Results shown in Figure 5D and Supplemental Figure 3 are from experiments performed on young seedlings grown on agar medium enclosed in sealed boxes. As discussed above, in an experiment performed on mature plants with fully expanded leaves, we also found that a SNP treatment (in this case, mature leaves of plants were sprayed with aqueous solutions containing SNP) reduced \textit{PRI} expression (monitored in this experiment using Northern analysis) in leaves of \textit{dnd1} plants (Figure 4A).
In this report, we have shown that a null mutation in CNGC2, a Ca\textsuperscript{2+}-conducting plasma membrane cation channel, results in a reduction in leaf Ca\textsuperscript{2+} levels during growth and development of dnd1 plants. We have associated the loss of function of this Ca\textsuperscript{2+} uptake pathway in dnd1 plants with a number of senescence-related phenotypes that are complemented by exogenous application of the NO donor SNP. In Figure 6, we identify another NO-related phenotype of dnd1 plants. Application of NO at high levels inhibits growth of, and is toxic to WT Arabidopsis (He et al., 2004) and tobacco (Morot-Gaudry-Talarmain et al., 2002) plants. In the experiment shown in Figure 6, WT and dnd1 seedlings were grown in sealed containers on growth medium containing varying concentrations of SNP. It is thought that in the light, SNP in aqueous solutions undergoes photochemical degradation to release gaseous NO (Floryszak-Wieczorek et al., 2006). Presumably, growth of seedlings in sealed, illuminated containers would allow for greater buildup of gaseous NO around (and in) plant tissue (as compared with spraying SNP on leaves or adding the NO donor to irrigation solution). Under these conditions, we note that increasing SNP in growth medium (to 100 µM) is lethal to WT seedlings while dnd1 seedlings are less affected (Figure 6). At 50 µM SNP, there is a visible difference between WT (more wilted appearance) and dnd1 seedlings (less affected) as well (Figure 6). This sensitivity of WT Arabidopsis seedlings to SNP addition to the growth medium we report here is similar to that shown by He et al. (2004). Current work notes the paucity of easily discerned plant phenotypes of CNGC loss-of-function mutants (Frietsch et al., 2007). Thus, our identification here of an NO-related phenotype of the dnd1 mutant could provide the basis for further study of CNGC-related effects of Ca\textsuperscript{2+} uptake inhibition on growth and development. One possible mechanism underlying the dnd1 phenotype shown in Figure 6 is as follows. Lower endogenous levels of NO present in dnd1 plants during growth (Figure 3) could allow for tissue in this mutant to be less sensitive (i.e., in an additive sense) to addition of exogenous NO.
Evidence presented in this manuscript depicts a model linking the function of the Ca$^{2+}$ conducting channel CNGC2 with downstream NO production and senescence programming. Prior work has shown that transitory Ca$^{2+}$ uptake into plant cells through CNGC2 initiated during pathogen defense signaling cascades involves downstream NO production. Here, we show that the same channel contributes to Ca$^{2+}$ uptake into the leaf during growth and development of the plant beyond this previously reported role in pathogen defense responses. The Ca$^{2+}$ uptake capability provided by CNGC2 apparently impacts NO generation during growth and development as well; null mutation of CNGC2 results in reduced endogenous NO level in *dnd1* plants as compared to WT plants. We find that exogenous application of an NO donor to *dnd1* plants reverses a number of senescence-related phenotypes. This link, between CNGC2-mediated uptake of Ca$^{2+}$ into leaf tissue and NO, presumably mediates leaf senescence development as a negative regulator. Therefore, this work provides new information about the molecular mechanism of plant senescence signaling.

We provide new experimental evidence indicating that *dnd1* mutants have reduced NO production during growth and development of the plant and associate this reduction in ‘basal’ NO level with the absence of a Ca$^{2+}$ uptake pathway which is operative during growth of the plant. Guo and Crawford (2005) also attributed the complementation of the early senescence phenotype of *atnoa1* mutants by SNP to a reversal (by the NO donor) of depressed NO generation during leaf senescence in these plants; they did not monitor the basal level of NO in these mutants. Mishina et al. (2007) also attributed the induction of early senescence by an NO-degrading treatment (NOD expression) to presumed changes in the basal level of this signaling molecule in leaves. In the work reported here, we observe that *dnd1* plants has a reduced endogenous level in leaves compared to WT, which supports the early senescence phenotypes in *dnd1* leaves that we found in this study.

Previous studies suggest that NO not only functions as a senescence signaling regulator (Mishina et al., 2007; Guo and Crawford, 2005) but also inhibits SA elevation during this process (Mishina et al., 2007). Work presented here is consistent with that
model. Application of SNP to dnd1 plants reduces their high SA level, as well as the transcript level of a marker gene (PRI) for SA generation.

In summary, our studies provide new genetic information linking Ca\(^{2+}\) uptake through CNGC2 channels and accumulation in leaves during the course of plant growth and development as a component of leaf senescence signaling. NO is also proposed to be involved in this signaling cascade. Results presented here are consistent with NO action as a negative regulator during the developmental progression to leaf senescence.
MATERIALS AND METHODS

Plant Material

Arabidopsis WT (Columbia ecotype) and dnd1 (Clough et al., 2000) plants were generally grown in a growth chamber on LP5 potting mix containing starter fertilizer (Sun Gro, Bellevue, WA) at 16 h light (100 μmol m⁻² s⁻¹ illumination)/8 h dark (72% relative humidity) and 22 °C. Seeds are vernalized at 4 °C in the dark for 2 d prior to use. During growth, plants were irrigated with half-strength Murashige and Skoog (MS) (Caisson, Rexburg, ID) solution 1-2 times to provide supplementary fertilizer. Alternatively, seeds were surface sterilized and spread on Petri dishes containing half-strength MS medium, 2.6 mM MES (adjusted to pH 5.7 with KOH), 1% sucrose, and 0.8% agar (see Ma et al., 2006). These seeds were germinated and grown in a growth chamber with a day (60–70 μmol m⁻² s⁻¹ illumination)/night cycle of 16/8 h at 25 °C. Rosette leaves from plants were used for experiments unless noted otherwise in Figure legends. In summary, leaf material obtained from plants grown on solid medium were used for experiments shown in Figure 1A-B, Figure 2D, Figure 5D, Figure 6, and Supplemental Figure 3. Plants grown using hydroponic culture were used for experiments shown in Figure 1C-D (for details see following sections). For all other experiments, leaves were harvested from plants grown on soil.

As characterized by Clough et al. (2000), the dnd1 Arabidopsis genotype is homozygous for a null mutation in the gene (At5g15410) encoding CNGC2. The dnd1 allele contains a G to A point mutation that creates a stop codon in exon 3 at Trp290, generating a severely truncated and nonfunctional CNGC2 coding sequence. All chemicals were purchased from Sigma (St. Louis, MO) unless noted otherwise.

Leaf Ca²⁺

WT and dnd1 plants were grown either on solid agar medium or on liquid nutrient solution (hydroponic culture). For growth on solid medium (experiments 1 and 2 in Figure 1), 15 seeds were germinated/ Petri plate on medium containing 0.8% w/v agar,
1 % (w/v) sucrose, 2.5 mM MES (pH 5.7), half-strength MS medium with either 1.5 mM Ca\(^{2+}\) (‘low Ca\(^{2+}\)’ treatment) or 20 mM (‘high Ca\(^{2+}\)’ treatment) final concentration CaCl\(_2\)\(\cdot\)2H\(_2\)O. Shoots (tissue growing above the solid medium) were harvested after three weeks. For hydroponic culture (experiments 3 and 4 in Figure 1), seedlings (maximum of 30/tank) were grown on ~5 cm length rock wool plugs extending into 18 L tanks containing 1.25 mM KNO\(_3\), 1.5 mM (‘low Ca\(^{2+}\)’ treatment) Ca(NO\(_3\))\(_2\)\(\cdot\)4H\(_2\)O, 0.75 mM MgSO\(_4\), 0.5 mM KH\(_2\)PO\(_4\), 72 µM EDTA (ferric sodium salt), 50 µM KCl, 50 µM H\(_3\)BO\(_3\), 10 µM MnSO\(_4\), 2 µM ZnSO\(_4\), 1.5 µM CuSO\(_4\), and 0.525 µM Na\(_2\)MoO\(_4\) with constant aeration. In the hydroponic culture for the ‘very low Ca\(^{2+}\)’ treatment, the medium contained 0.75 mM Ca(NO\(_3\))\(_2\)\(\cdot\)4H\(_2\)O, KNO\(_3\) was increased to 2.75 mM, and all the other components were added at the same concentration as stated above. Water was added every other day to maintain the level of solution in tanks, and leaves were harvested after four (experiment 3) or seven (experiment 4) weeks of growth. Harvested leaves (hydroponics) or shoots (from agar plates) were dried at 70 °C for 2-3 d prior to tissueashing and analysis of Ca\(^{2+}\) (using an inductively coupled plasma mass optical emission spectrometer) by the NSF-funded Purdue Ionomics Facility as described (Salt, 2004). A minimum of 200 mg fresh weight of tissue was used for each tissue sample. Replicate tissue samples were taken from different plates in the case of the solid medium experiment, and from different plants in the case of the hydroponic experiment.

**Dark-induced leaf senescence**

The darkness-induced leaf senescence assay we used was a method adapted from Ülker et al. (2007). In brief, leaves were detached from 5-week-old plants and floated on water or water containing 100 µM Gd\(^{3+}\) in Petri dishes as described in Figure legends. The first true leaf at the bottom of the shoot (arising after the cotyledons) was counted as the oldest leaf. Leaves with same age were used to ensure the accuracy of the experiments. Petri dishes were wrapped with aluminum foil and kept at 22 °C. Pictures were taken after 3-5 d as indicated in Figure legends.
Lipid peroxidation

The extent of lipid peroxidation in leaves was evaluated by measuring MDA formation as described by Guo and Crawford (2005). For these studies, 3-week-old plants were used except for the experiment shown in Supplemental Figure 1; in this case plants were 5-week-old. In brief, each leaf sample was either ground in chilled extraction buffer (containing 0.25 % (w/v) thiobarbituric acid in 10 % (w/v) trichloroacetic acid (Fisher Scientific)) directly or first ground in liquid nitrogen followed by resuspension in extraction buffer. The homogenates were incubated in a water bath (90 °C) for 20 min. Heated homogenates were then equilibrated to room temperature, and centrifuged (12,000 X g for 15 min). Supernatants were decanted for optical density measurement at A$_{532}$ and A$_{600}$ for MDA determination using an extinction coefficient presented by Heath and Packer (1968). Effect of an exogenous NO donor on lipid peroxidation was evaluated. In this case, WT and dnd1 plants were treated with the NO donor by spraying leaves (once per day) with water containing 0 or 100 µM SNP for 2 d as described in Beligni and Lamattina (1999). When lipid peroxidation was monitored during dark-induced leaf senescence, plants were treated as noted above. Samples were harvested for the MDA measurement 0-3 d after initiating the dark treatment as indicated in Figure legends.

Gene expression (RNA extraction, semi-quantitative RT-PCR, quantitative real-time PCR and Northern blots)

For experiments shown in Figures 2D, 5D and Supplemental Figure 3, three-week-old plants were used. Five-week-old plants were used for experiments shown in Figures 2F, 4B and Supplemental Figure 2. For experiments involving application of an exogenous NO donor, three-week-old seedlings were transplanted to clear polycarbonate boxes containing half strength MS medium (with either 0 or 50 µM SNP). Five d after transplanting, leaves were harvested and frozen in liquid nitrogen for RNA extraction. Dark-induced leaf senescence experiment was performed as described in the above paragraph. Samples were harvested for the RNA extraction 0-4 d as indicated in Figure
Total RNA was isolated from leaf samples (ground in liquid nitrogen) using the RNA kit (Qiagen, Valencia, CA) following the supplier’s protocol. Genomic DNA contamination was removed using the DNA-free reagent (Ambion, Austin, TX) following the manufacturer’s instruction. First strand cDNA was synthesized in 20 µL using 1 µg RNA, and reagents from RETROscript Kit (Ambion) following the manufacturers’ instructions. Subsequent PCR experiments were performed using 1.5 µL first strand cDNA as template. Arabidopsis tubulin β-subunit (At5g44340) was used as the internal standard with the following gene specific primers (F: 5’-ACGTATCGATGTCTATTTCACAACGA-3’ and R: 5’-ATAATCGTAGAGAGCCTCATTGTCC-3’) as described in Ülker et al. (2007). Primers for PRI (At2g14610) (F: 5’-TCGTCTTTGTAGCTTTGTAGGTG-3’ and R: 5’-TTCTATTAGTATGGCTTCTCGTCA-3’) and WRKY70 (At3g56400) (F: 5’TAATGGGATACTAATAAGCAGAAAAAGC 3’, R: 5’-CAGATAGATTCCAACATGAACATGAAG-3’) were described in Ülker et al. (2007). Primers for RLK5 (At4g23140) (F: 5’-TAAAGCAAGTAACATTCTCCTACATGC-3’, R: 5’-AGCATGAAGTTTAAGGTCATATTTAGC-3’) were described in Galon et al. (2008). Primers for gene At5g10760 were designed as follows: F: 5’-CTTGTTGTGAAGAAGGGAGG-3’, R: 5’-CCGCAACACAAAAGTAAACATCCTCCA-3’. No DNA contamination was found in the RNA sample after DNA-free reagent (Ambion) treatment.

Quantitative real-time PCR was using LightCycler FastStart DNA MasterPLUS SYBR Green I Kit (Roche Applied Science, Mannheim, Germany) or SYBR Green PCR Core Reagents Mix (Applied Biosystems, Carlsbad, CA), according to the manufacturer’s manual. The level of gene transcript accumulation was normalized to an internal standard tubulin β-subunit (At5g44340). Primers used for real-time PCR were either same as described above or as following: SAG12 (At5G45890): F:5’-GCGGATGTGAAGGAGGAAAA-3’, R:5’-CAATGCGTTCGACGTGTTTT-3’. tubulin β-subunit (At5g44340): F: 5’-TGTTTCGTTTCATGTGTTTGTTTCAACAAATCCA-3’. R: 5’-ACACGCAAAAGTGTTAACAATCCA-3’.
For Northern blot analysis, RNA was isolated from leaves of plants sprayed with water containing 0 or 100 µM SNP once daily for 2 d as described by Beligni and Lamattina (1999) using TRI REAGENT® (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s instructions. RNA blotting and probe labeling protocols followed the procedures described in Mercier et al. (2004). Primers for the PRI probes were amplified using primers 5’-GTAGGTGCTCTTGTCTTCCTCC-3’, 5’-CACATAATTCCACGAGGATC-3’ as described in Jurkowski et al. (2004).

**H₂O₂ and cell death in leaves**

Rosette leaves of 4- to 6-week-old WT and dnd1 plants were irrigated with half strength MS solution containing 100 µM SNP or half strength MS solution alone (control) for 7 to 10 d as described in Ali et al. (2007). After treatment, the leaves were detached from the plant, and used for either *in vivo* H₂O₂ detection or assay of cell death using Trypan Blue staining. The H₂O₂ detection assay was performed as previously described by Kwak et al. (2003). A 3,3’-diaminobenzidine (DAB, Sigma) stain was vacuum infiltrated into detached leaves for 1-2 min; leaves were then placed into a humid plastic box and left overnight. An ethanol:glycerol:85% lactic acid (3:1:1) fixation solution was added to the leaves, which were then shaken at 50 rpm for 24-48 h until pigments were completely removed from the leaf tissue. Trypan Blue staining was performed as previously described in Jirage et al. (2001). In brief, detached leaves were boiled in a staining solution containing Trypan Blue (Sigma) at a final working concentration of 250 µg/mL in 1:1:1:1 (v/v/v/v) water:1 M phenol:glycerol:85% lactic acid. Images were captured with an EPSON 1660 Perfection Photo Scanner (Long Beach, CA, USA) using Adobe Photoshop Imaging software (San Jose, CA, USA). All microscopic photographs were taken with an inverted Olympus IX70 microscope (Olympus, Melville, NY).

**Endogenous NO in leaves**
The method used to measure endogenous NO in leaves was adapted from that described by Lamattina and colleagues (Martin et al., 2009; Graziano and Lamattina, 2007). NO was monitored using the NO-specific fluorescent dye 3-amino, 4-aminomethyl-2',7'-difluorofluorescein diacetate (DAF-FM DA) (Calbiochem). The use of DAF dye to measure NO in plant leaves has been used in conjunction with alternative methods for NO quantification in prior studies. In this prior work, effects of treatments on leaf NO as monitored with DAF fluorescence were comparable to NO analyses using alternative methods. For example, NO elevations due to exposure of leaves to lipopolysaccharide were detected similarly using either DAF fluorescence or electron paramagnetic resonance (Zeidler et al., 2004).

Leaves were detached from 4- to 5-week-old WT and dnd1 plants and weighed (each sample is around 100 mg). Each individual sample was then vacuum infiltrated with or without 10 μM DAF-FM DA (NO detection dye) in 20 mM HEPES/NaOH (pH 7.5) buffer. Leaves were kept in this solution for 10 min after vacuum infiltration. The samples were then washed three times (shaking 1 min each time) in Petri dishes containing 20 mM of HEPES/NaOH (pH 7.5) buffer. After washing, samples were blotted to remove excess buffer, and then homogenized in 0.5 mL of 20 mM HEPES/NaOH (pH 7.5) buffer. The extracts were then centrifuged at 15,000 g at 4 °C for 10 min. 100 μL of collected supernatant was added to the well of a 96-well microplate (black walls and clear bottom), and fluorescence signals were quantified using a FLUOstar Optima microplate reader (BMG Labtech) at excitation and emission wavelengths of 485 and 520 nm, respectively. Endogenous NO content was ascertained by subtracting a background signal value from the individual sample. Background signal was typically obtained by measuring fluorescence signals in WT and dnd1 samples infiltrated with 20 mM of HEPES/NaOH (pH 7.5) buffer alone.

**Leaf salicylic acid**

Eight-week-old plants were treated with an NO donor by spraying leaves with water containing 0 or 100 μM SNP daily for 2 d as described in Beligni and Lamattina (1999). SA and its glucoside were quantified using gas chromatography-mass
spectrometry. 100 mg of frozen leaf tissue were extracted twice with 800 μL acetone: 50 mM citric acid (7:3, v/v) in 2 mL Fast Prep tubes containing ceramic beads using a FastPrep FP 120 tissue homogenizer (Qbiogene, Carlsbad, CA). Radiolabeled [²H₆] SA (CDN Isotopes, Point-Claire, QC, Canada) was added to leaf samples as an internal standard. After evaporation of the acetone under vacuum the aqueous solutions were extracted twice with 750 μL of diethyl ether. SA-glucoside was extracted from the remaining aqueous solution after acidification with 5 μL of HCl and hydrolysis at 90 °C for 1 h by diethyl-ether extraction. All samples were then loaded on 1 mL Supelclean LC-NH₂ SPE columns (Supelco, Bellefonte, PA). After washing with 1.2 mL chloroform: 2-propanol (2:1, v/v) compounds were eluted with 1.5 mL diethyl-ether: formic acid (98:2, v/v). The eluates were then evaporated to dryness under a stream of N₂, dissolved in 100 μL dichlormethane: methanol (8:2, v/v) and derivatized with 2 μL Trimethylsilyldiazomethane (Aldrich, Oakville, ON, Canada) for 20 min. The reaction was stopped by adding 2 μL of 2 M acetic acid in hexane (Schmelz et al., 2004). The resulting methyl esters of SA were analyzed by gas chromatography-mass spectrometry (6890N GC connected to a 5975 mass selective detector, Agilent Technologies, Palo Alto, CA) in isobutene chemical ionization mode following the specifications of Schmelz et al. (2004). The methyl esters were measured using selected-ion monitoring with m/z 153 (SA) and m/z 157 ([²H₆] SA). Results are presented as total (conjugated and free) SA.
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FIGURE LEGENDS

Figure 1. Leaf Ca\(^{2+}\) level is reduced in \textit{dnd1} plants as compared to WT plants. Results from plants grown on solid medium (experiments 1 and 2) and hydroponic medium (experiment 3 and 4) are shown. The ‘very low’, ‘low’, and ‘high’ Ca\(^{2+}\) treatments correspond to 0.75, 1.5, and 20 mM Ca\(^{2+}\) in the medium respectively. Results for WT (open bars) and \textit{dnd1} (filled bars) plants are shown as means ± SE (\(n > 3\)). ANOVA analysis was used to evaluate means separation between paired WT and \textit{dnd1} measurements (for each experiment at a specific growth medium Ca\(^{2+}\) level). Significant differences (at \(p \leq 0.05\) and \(p \leq 0.01\), respectively) are indicated by a ‘*’ or ‘**’ above filled bars. The low Ca\(^{2+}\) treatment corresponds to the ‘standard’ Ca\(^{2+}\) level in half strength Murashige and Skoog (MS) solid medium typically used for growth of Arabidopsis on agar plates. The ‘high Ca\(^{2+}\)’ treatment corresponds to that used by Chan et al. (2003) for growth of WT and \textit{dnd1} plants. The ‘very low’ Ca\(^{2+}\) treatment in experiment 4 (i.e. 0.75 mM in hydroponic nutrient solution) corresponds to a 50% reduction from that found in standard nutrient solutions. Plants grown on liquid hydroponic culture (experiments 3 and 4) had higher levels of leaf Ca\(^{2+}\) than that found in plants grown on solid medium (experiments 1 and 2). This could be due to either larger root systems in hydroponically grown plants or the increased availability of nutrients in a constantly-mixed hydroponic solution as compared to solid medium, where roots could deplete Ca\(^{2+}\) in localized zones. Leaves were harvested from plants grown for 3 (experiments 1 and 2), 4 (experiment 3) or 7 (experiment 4) weeks, respectively.

Figure 2. Leaves of \textit{dnd1} plants display earlier senescence phenotypes as compared to leaves of WT plants. (A) \textit{dnd1} plants show earlier natural senescence; i.e. leaves begin turning yellow at the tip (highlighted by arrows). Insert shows an enlarged image of a portion from one leaf of the \textit{dnd1} plant. (B) Leaves detached from WT plants (top and center rows) and \textit{dnd1} plants (bottom row) were incubated on water, or water containing 100 µM Gd\(^{3+}\) (center row). From left to right, lanes 1-4 show leaves after 0, 3, 4, and 5 d in darkness, respectively. Counting from the first true leaf, leaves 3-8 of seedlings were used for this assay. (C) Images are shown of individual detached leaf (in this case, leaves
shown are either leaf 1 or 2) of the seedlings used for the experiment shown in (B). Lanes 1-3 correspond to 0, 3, and 4 d in darkness treatment, respectively. (D) RT-PCR products generated with primers corresponding to SAG genes, and RNA prepared from leaves (the entire rosette) of WT or dnd1 plants as template were subjected to agarose gel electrophoresis. A band corresponding to tubulin is shown as a loading control. (E) MDA levels in (rosette) leaves of WT and dnd1 plants (expressed per unit leaf fresh weight (FW)). Results are presented as mean (n = 3) ± SE. ANOVA evaluation of means separation indicated differences between WT and dnd1 MDA levels were significant at p ≤ 0.01. Similar results were found when the experiment was repeated three times. (F) Quantitative real-time PCR analysis of SAG12 transcript accumulation (relative to tubulin transcript) in WT detached leaves (leaves 3-5) left in the dark (0-4 d) on water or Gd³⁺. Results are shown as means ± SE (n = 3). ANOVA analysis was used to evaluate means separation at each time point. Significant differences (at p ≤ 0.01) are indicated by a ‘***’ above symbols.

**Figure 3.** Endogenous leaf NO is reduced in dnd1 plants as compared to WT plants. Genotype means of arbitrary fluorescence units (n = 3) ± SE are shown for three independent experiments (A-C). A ‘***’ above a bar representing NO levels in dnd1 plants indicates the difference from the level found in WT leaves is significant at p ≤ 0.01 for an individual experiment. ANOVA (paired T-Test) evaluation of means separation between WT and dnd1 genotypes for the pooled values from all three experiments indicated the genotype differences were significant at p ≤ 0.01.

**Figure 4.** PRI expression (A) and total salicylic acid (B) in leaves of dnd1 plants treated with water (-SNP) or 100 µM SNP (+SNP). PRI expression was monitored using Northern blot hybridization; loading controls show the 23S ribosomal RNA (rRNA) band from the corresponding agarose gel stained with ethidium bromide. The experiment shown in (A) was repeated a total of two times. Results in (B) are presented as means (n = 3) ± SE.
Figure 5. Application of NO donor SNP rescues senescence associated phenotypes in *dnd1* plants. (A) Endogenous H$_2$O$_2$ levels in leaves of WT (left panels) and *dnd1* (right panels) plants were detected using 3,3’-diaminobenzidine (DAB) staining. Leaves are shown from plants treated with water or with SNP are shown in upper and bottom panels, respectively. (B) Necrosis, monitored using Trypan blue staining, in leaf tissue of WT and *dnd1* plants treated with water (-SNP) or 100 µM SNP (+SNP). Dead cells become blue after staining. This experiment was repeated three times. (C) Lipid peroxidation (MDA level) in leaf tissue of WT and *dnd1* plants treated with water (-SNP) (dark bars) or 100 µM SNP (+SNP) (light bars). Results are presented as means (n = 3) ± SE. ANOVA evaluation of means separation between –SNP and +SNP treatments indicated a significant difference for *dnd1* leaves (p ≤ 0.05; indicated with a ‘*’ above bar representing the ‘+ SNP’ treatment) and no significant difference for WT leaves. (D) Effect of (50 µM) SNP on SAG transcript accumulation in *dnd1* leaves was analyzed by semi-quantitative RT-PCR. This experiment was repeated three times.

Figure 6. WT seedlings are more sensitive than *dnd1* seedlings to exogenous NO. WT and *dnd1* seedlings were transplanted into (covered) Magenta boxes with half strength MS medium containing 0, 50, or 100 µM SNP. Pictures were taken 4 d after transplanting. This experiment was repeated three times with similar results.

Supplemental Figure 1. MDA levels in leaves detached from WT plants and floated in the dark on water or 100 µM Gd$^{3+}$ for 0-3 d. Leaves 3-5 of seedlings were used for this assay. Results are presented as mean percentage change (from day 0) for each genotype (n = 3) ± SE. ANOVA analysis was used to evaluate means separation between WT and *dnd1* at each time point. Significant differences (at p ≤ 0.05) are indicated by a ‘*’ above symbols.

Supplemental Figure 2. *SAG12* transcript accumulation in WT and *dnd1* detached leaves (kept in the dark on water for 0-2 d) was analyzed by semi-quantitative RT-PCR. *tubulin* is shown as a loading control. Leaf 5 of seedlings was used for this assay.
Supplemental Figure 3. Quantitative real-time PCR analysis of PRI transcript accumulation (relative to tubulin transcript) in leaves of dnd1 and WT plants treated with 0 or 50 µM SNP as described in Figure 5D.
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