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# Genetic Screening for Plant Cell Death Suppressors and Their Functional Analysis in Plants

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Bax, a mammalian pro-apoptotic member of the Bcl-2 family, induces cell death when expressed in yeast. To investigate whether *Bax* expression can induce cell death in plant, we produced transgenic *Arabidopsis* plants that contained murine *Bax* cDNA under control of a glucocorticoid-inducible promoter. Transgenic plants treated with dexamethasone, a strong synthetic glucocorticoid, induced Bax accumulation and cell death, suggesting that some elements of cell death mechanism by Bax may be conserved among various organisms. Therefore, we developed novel yeast genetic system, and cloned several Plant Bax Inhibitors (PBIs). Here, we report the function of two PBIs in detail. PBI1 is ascorbate peroxidase (sAPX). Fluorescence method of dihydrorhodamine123 oxidation revealed that expression of Bax in yeast cells generated reactive oxygen species (ROS), and which was greatly reduced by co-expression with sAPX. These results suggest that sAPX inhibits the generation of ROS by Bax, which in turn suppresses Bax-induced cell death in yeast. *PBI2* encodes nucleoside diphosphate kinase (NDPK). ROS stress strongly induces the expression of the NDPK2 gene in *Arabidopsis thaliana* (*AtNDPK2*). Transgenic plants overexpressing *AtNDPK2* have lower levels of ROS than wildtype plants. Mutants lacking *AtNDPK2* had higher levels of ROS than wildtype. $H_{2O2}$ treatment induced the phosphorylation of two endogenous proteins whose molecular weights suggested they are AtMPK3 and AtMPK6. In the absence of H2O2 treatment, phosphorylation of these proteins was slightly elevated in plants overexpressing *AtNDPK2* but markedly decreased in the *AtNDPK2* deletion mutant. Yeast two-hybrid and *in vitro* protein pull-down assays revealed that AtNDPK2 specifically interacts with AtMPK3 and AtMPK6. Furthermore, AtNDPK2 also enhances the MBP phosphorylation activity of AtMPK3 *in vitro*. Finally, constitutive overexpression of AtNDPK2 in *Arabidopsis* plants conferred an enhanced tolerance to multiple environmental stresses

that elicit ROS accumulation *in situ*. Thus, AtNDPK2 appears to play a novel regulatory role in H2O2-mediated MAPK signaling in plants.

Key words: Cell death, Bax, reactive oxygen species, nucleoside diphosphate kinase, stress tolerance

## INTRODUCTION

Programmed cell death (PCD) or apoptosis, an evolutionarily conserved form of cell suicide, occurs routinely during organism development, and in response to environmental factor. This process is important for eliminating unwanted, damaged, infected or useless cells that would otherwise cause inflammation of the surrounding cells with their cytoplasmic contents (Steller, 1995). In plants, PCD is essential for normal reproductive and vegetative development, specifically, gamete development, sex determination, embryogenesis, leaf abscission, formation of tracheary element, aerenchyma formation, and hypersensitive response to environmental stress (Dangl et al., 2000). Although very little is known about the mechanism of PCD in plants, it is suggested that the proper regulation of this process involves genetic control (Dangl et al., 2000).

In animals, apoptosis is under genetic control and the signaling pathways and genes involved in apoptosis have been extensively studied (Williams and Smith, 1993). The Bcl-2 family of proteins are important regulators of cellular apoptosis (Gross et al., 1999). Bcl-2 proteins promote either cell survival (Bcl-SL, Bcl-2, Bcl-W, Bcl-$X_L$, Bfl-1 Mcl-1, A1, Brag-1 and A1) or cell death (Bax, Bak, Bcl-XS, Bid, Bik, Hrk, and Bok). Several of these regulatory proteins are located in the outer membrane of mitochondria, and play crucial roles in apoptosis by releasing apoptogenic factors such as cytochrome c and apoptosis-inducing factor (AIF) from intermembrane space into cytoplasm, which in turn activate caspases, hallmarks of apoptotic execution. While pro-apoptotic members of the Bcl-2 family induce apoptosis-associated mitochondrial release of both cytochrome c and AIF, the anti-apoptotic members counteract this process. Ultimately, the ratio or equilibrium between pro- and anti-apoptotic proteins determines the susceptibility of a particular cell to apoptosis (Gross et al., 1999).

The expression of Bax in yeast induces apoptosis, which can be suppressed by co-expression

with anti-apoptotic members of the same protein family, Bcl-2 or Bcl-$X_L$ (Hanada et al. 1995; Greenhalf et al., 1996; Jurgensmeier et al., 1997). Yeast mitochondria seems to be involved in Bax-induced cell death in the similar ways as in mammalian cells, involving release of cytochrome c (Manon et al., 1997) and alterations in mitochondrial membrane potential (Minn et al., 1999). The phenotype of cell death promoted by Bax in tobacco plant closely resembles hypersensitive response, a type of PCD in plants induced by tobacco mosaic virus, signify that expression of Bax in plants is also lethal (Lacomme and Cruz, 1999).

Observations on Bax-induced cell death in animals, yeasts and plants suggest that some elements of this mechanism may be conserved among various organisms. We employed yeast, a powerful genetic tool, to identify the molecular determinants of Bax-induced apoptosis in plants. Soybean cDNA library was co-transformed with the *Bax* gene into yeast cells, and over-expressed genes that could suppress Bax-induced cell death were isolated.From the repertoire of the Bax-inhibiting proteins obtained, we characterized sAPX and NDPK in detail.

**Expression of Bax in Arabidopsis induces cell death**

To investigate the pro-apoptotic property properties of Bax in plants, we generated a transgenic strain of *Arabidopsis* plants that expressing expresses Bax under the control of a glucocorticoid-inducible promoter. The expression of Bax in these transgenic plants was induced by dipping only the leaf petioles of leaves from $T_1$ plants into dexmetason a dexamethasone solution (20 mM) and then incubating them under the light for 2-3 days. The Bax expression level of Bax in each transgenic plant after following inducing induction with dexamethasonedexmetason solution was determined by western blotting using with a Bax monoclonal antibody (data not shown). The Bax expression levels are consistent with the severity of Bax-mediated cell death. Since dexamethasonedexmetason was supplied delivered through vescular vascular tissue, cells around surrounding veins were killed dead first and thenfollowed by cell death was spreadspreading to the whole leaf in severe cases. In addition, when dexamethasonedexmetason was spreading on the whole plants, first the leaves turned into yellow and then they completely collapsed, Subsequently, and eventuallythe whole plant was deaddied due to the systemic movement of dexmetason dexamethasone (Fig. 1).

**Screening of plant cell death suppressors using yeast genetic system**

Observations on Bax-induced cell death in animals, yeasts and plants suggest that some elements of this mechanism may be conserved among various organisms. Therefore, we employed yeast, a powerful genetic tool, to identify the molecular determinants of Bax-induced apoptosis in plants (Moon et al., 2002). *Arabidopsis* or soybean cDNA library was co-transformed with Bax into an yeast strain W303-1a, and transformants were selected on glucose containing medium. For conditional expression of Bax in yeast, the gene was placed under the control of a GAL1 promoter that allowed specific expression of the protein when cells were grown in galactose-containing medium instead of dextrose as carbon source. Approximately, 2 x $10^5$ independent transformants were collected. After extensive washing in water, transformants were plated onto a galactose-containing medium and plasmids from selected surviving clones were isolated and analyzed by restriction mapping. Based on their restriction patterns, plasmid inserts corresponding to five different loci were designated *PBI1, PBI2, PBI3, PBI4*, and *PBI5* (for plant Bax inhibitor). To verify that PBIs suppress Bax-induced cell death in yeast, colony formation was observed on a galactose-based medium (Fig. 2A). Colonies formed by cells harboring both pGilda-Bax and pADGal4-2.1-PBI on glucose-based medium were detected with approximately the same efficiency as control transformants containing plasmid pGilda-Bax with an empty vector. However, while transformants containing Bax with an empty vector on galactose medium induced complete inhibition of colony formation, those containing Bax with PBI restored cell growth. Immunoblot analyses were performed to examine whether PBI affects the expression levels of Bax protein (Fig. 2B). No Bax protein was detected in glucose-based medium. Upon transfer of cells from glucose-based medium (in which the GAL1 promoter is repressed) to galactose-based medium, Bax protein accumulated to easily detectable levels within 12 hrs in the cells containing plasmid pGilda-Bax without or with PBI. These findings suggest that PBI is one of the specific proteins in plants that suppress Bax-induced cell death in yeast.

***PBI1* encodes ascorbate peroxidase (sAPX) : sAPX suppresses Bax-induced apoptosis in yeast by inhibiting ROS**

Increasing evidence suggests that ROS are effectors of PCD in animals and plants (Jabs,

1999),and play a role in stress adaptation in prokaryotes (Hochman, 1997).In yeast, depletion of free radical generation during hypoxia prevented PCD induced by the mutant cdc48 S565G allele or over-expression of Bax (Madeo et al., 1999). Since *PBI1* encodes sAPX, an antioxidant protein, we reasoned that Bax may generate ROS and this is suppressed by sAPX. Therefore, we analyzed the production of ROS during Bax-induced cell death in yeast, using dihydrorhodamine123 and DCFH-DA. Upon oxidation by ROS, non-fluorescent dihydrorhodamine123 becomes a fluorescent chromopore, rhodamine123, and DCFH-DA is deacylated and oxidized to fluorescent compound dichlorofluorescein. We noted that more than 35% cells exhibited fluorescence when yeast cells harboring pGilda-Bax were incubated with dihydrorhodamine123 (Fig. 3A middle). However, most of the corresponding wild-type cells, and cells expressing both Bax and PBIs exhibited no fluorescence, appearing dark against faint background fluorescence (Fig. 3A left and right). The production of ROS by Bax in yeast cells was further confirmed by flow cytometry analyses with dihydrorhodamine123. Yeast cells harboring pGilda-Bax accumulated a large number of oxygen radicals, which was significantly inhibited by co-expression with sAPX (Fig. 3B). Similar results were obtained on ROS generation by DCFH-DA (data not shown). Our results suggest that the antioxidant capacity of sAPX blocks the hyper-production of intracellular ROS promoted by Bax.

***PBI2* encodes nucleoside diphosphate kinase (NDPK): AtNDPK2 interacts with two oxidative stress-activated MAPKsto regulate cellular redox state and enhances multiple stress tolerance in transgenic plants**

NDPK (EC 2.7.4.6) is believed to be a housekeeping enzyme that maintains the intracellular levels of all (d)NTPs used in biosynthesis except ATP. However, increasing lines of evidence suggest that NDPK also plays a significant role in signal transduction pathways (Otero, 2000). In animals, NDPKs play important roles in vital processes such as the control of cell proliferation, regulation of transcription, and protein phosphotransferase activity (Cipollini et al., 1997 Engel et al., 1998 Wagner and Vu, 2000). In plants, it is associated with the phytochrome A response, UV-B signaling, heat stress and growth (Choi et al., 1999 Galvis et al., 2001; Zimmermann et al., 1999; Pan et al., 2000). Hence, NDPK is strongly implicated in the regulation of cellular protein functions, possibly through its phosphotransferase activity

(Galviset al., 2001; Barthel and Walker 1999; Mesnildrey et al., 1997). However, why NDPKs have such diverse cellular functions and how NDPKs are regulated in response to various cellular processes is still poorly understood.

To determine the physiological role of *NDPKs* in ROS mediated signaling in plants, we used the *Arabidopsis* genetic system (Moon et al., 2003). We found that (1) $H_2O_2$ induces the transient expression of *AtNDPK2*, (2) AtNDPK2 specifically interacts with two $H_2O_2$-activated MAPKs, AtMPK3 and AtMPK6, as well as enhancing the MBP phosphorylation ability of AtMPK3, and (3) overexpression of *AtNDPK2* in plants leads to decreased constitutive ROS levels and enhanced tolerance to multiple environmental stresses that elicit ROS accumulation *in situ* (Fig. 4). Although how these proteins interact and function together remainsareto be determined in planta, our observations suggest that AtNDPK2 is a novel component of a pathway specific to $H_2O_2$-activated MAPK signaling in plants. We hypothesize that the stress tolerance function induction of AtNDPK2 resides its ability byto high redox state by environmental stress and then down-regulateion of cellular redox states, caused by environmental stress, via specific activation of the $H_2O_2$-activated MAPKs, AtMPK3 and AtMPK6 by AtNDPK2 underlies its stress tolerance function. Since AtNDPK2 was previously identified as a phytochorome-mediated light signal mediator (Choi et al., 1999), this model also suggests that light signals maymaymodulate stress responses, in addition to and protecting s plants from stress.

Generation of ROS in plants has been is implicated inwith abiotic and biotic stress responses, in which the level of ROS is an important cellular regulator for stress response as well as oxidative cell death. Therefore, it is crucial that plants maintain anthe adequate level of cellular redox state to make proper stress responses and overcome the stress. The environmental stress response is accelerated under light, although there is no direct evidence that light signals are is mediated by ROS in plants. For instance, phytochrome mediated light signaling modulates cold/drought-induced gene expression (Kim et al., 2002) and SA-induced PR gene expression as well as the hypersensitive response to pathogens (Genoud et al , 1998; Genoud et al., 2002). In contrast, antioxidant deficient transgenic plants induce lesions under strong high light (Chamnongpol et al., 1996; Mach et al., 2001), suggesting that light may be required for the amplification of an adequate ROS response of sufficient

amplitude to induce to allow stress-mediated cell death. This It implies that plants need to activate ROS scavenger enzymes for to make normal growth and development under stronghigh light.

The multiple stress tolerance of transgenic plants overexpressing *AtNDPK2* is similar to that of transgenic plants overexpressing the constitutively active deletion mutant of *ANP1* (Kovtun et al., 2000 Moon et al., 2003). Methyl viologen produces intracellular ROS by inhibiting photosynthesis and photorespiration, and is widely used as a source of superoxide radicals in studies related to photosynthesis (44). *ANP1* initiates the phosphorylation cascade that involves *AtMPK3* and *AtMPK6* in a ROS and light dependent manner (Kovtun et al., 2000). Dual localizations of AtNDPK2 to the nucleus and cytoplasm and its involvement in phytochrome A signaling and ROS dependent up regulation (Moon et al., 2003) suggests an important role for NDPK2 in the environmental stress associated with ROS generation (Choiet al., 1999, Zimmermann et al., 1999). These resultsstrongly indicate that AtNDPK2 may be an important upstream signaling component of the AtMPK3 and AtMPK6-mediated signaling cascade associated with stress tolerance in plants. How such multiple stress tolerance can arise from *AtNDPK2* overexpression is suggested by our cDNA microarray studies (Yang et al. 2003), which showed that *AtNDPK2* overexpression is associated with increased expression of a number of antioxidant genes, including peroxidase, glutathione reductase, glutathione transferase, thioredoxin reductase, peroxiredoxin and, protective genes encoding several heat shock proteins. Thus, we suggest that AtNDPK2 mediates multiple stress tolerance by signaling the transient expression of genes involved in antioxidant and protective functions possibly through activation of MAPK cascade.

## Conclusions

In this report, we demonstrate that a plant antioxidant gene functions as a suppressor of Bax-induced PCD in yeast cells. We propose that yeast *S. cerevisiae* is a powerful and ideal eukaryotic model system to identify plant genes involved in antioxidant and antiapoptotic function, and should facilitate clarification of the PCD mechanism conserved within various organisms.

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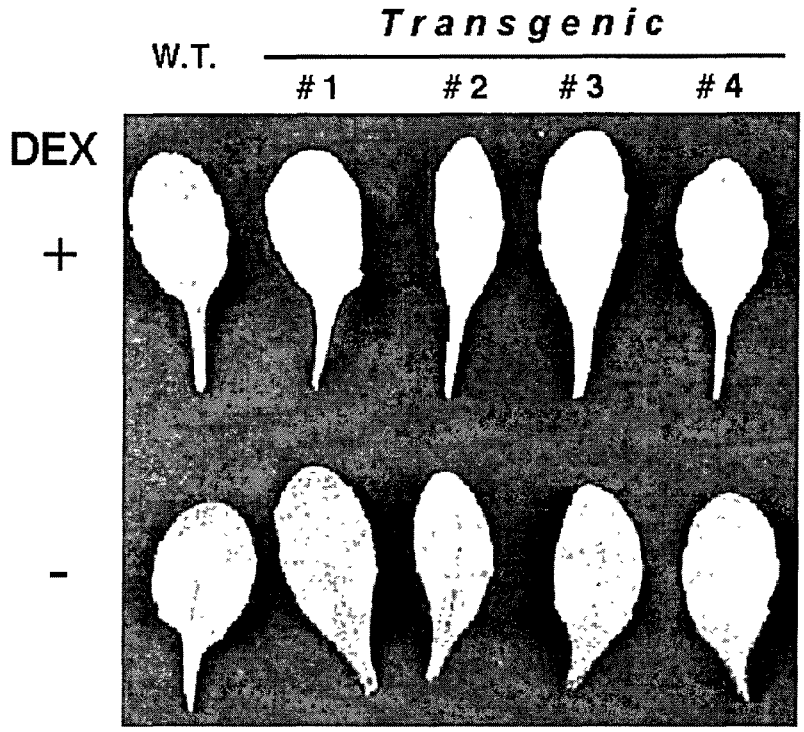


Fig. 1. Expression of Bax in *Arabidopsis* induces cell death. Expression of Bax in six T1 transgenic plants (Transgenic) was induced by dipping leaf petioles into 20 μM dexamethasone solution (DEX). Photographs were taken at 3 days after incubation. W. T., wildtype plant.

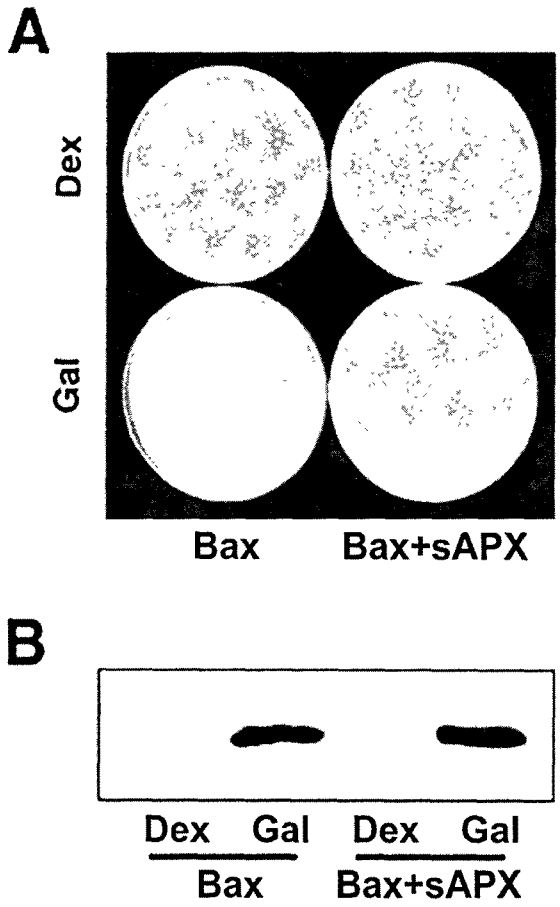


Fig. 2. PBI protein suppresses Bax-induced cell death in yeast. (A PBI-promoted resistance to Bax lethality. W303-1a cells harboring either pGilda-Bax/pADGal4-2.1 (left) or pGilda-Bax/pADGal4-2.1-PBI (right) were spread onto SD-glucose (Dex)- or SD-galactose (Gal)-containing plates. Photographs were taken after culturing at 30℃ for 2 days. (B) Western blot analyses. W303-1a transformants used in (A) containing pGilda-Bax/pADGal4-2.1 (Bax) or pGilda-Bax/pADGal4-2.1-PBI (Bax+ PBI)were grown in glucose-containing medium (Dex), subsequently transferred to galactose-containing medium (Gal) and cultured for 12 hrs. Total protein extracts (20 μg/lane) were subjected to SDS-PAGE and immunoblot analyses were performed, using anti-mouse Bax antiserum.

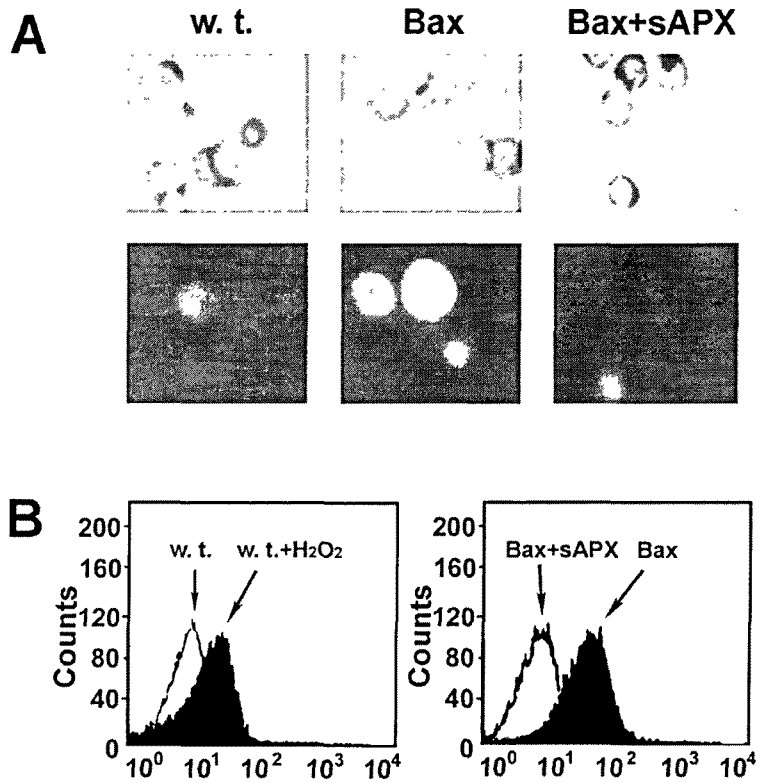


Fig. 3. Over-expression of PBI suppresses generation of ROS by Bax. (A) Microscopy analyses. Wild-type W303-1a cells, and W303-1a cells harboring either pGilda-Bax/pADGal4-2.1 or pGilda-Bax/pADGal4-2.1-PBI constructs were grown in galactose medium for 12 hrs. Following this, cells were incubated with 50 μM dihydrorhodamine123 for 2 hrs and subjected to microscopy. Fluorescence data after incubation with dihydrorhodamine123(lower) and the corresponding phase contrast display (upper) are depicted. (B) Flow cytometric analyses. W303-1a transformants containing pGilda-Bax/pADGal4-2.1 (Bax) or pGilda-Bax/pADGal4-2.1-PBI (Bax+PBI) were grown in galactose medium for 12 hrs. Cells were further incubated with 50 μM dihydrohodamine123 for 2 hrs for flow cytometric analyses (right panel). Wild-type W303-1a cultures grown in media without or with 1 mM $H_2O_2$ for 15 minwas employed as a control (left panel).

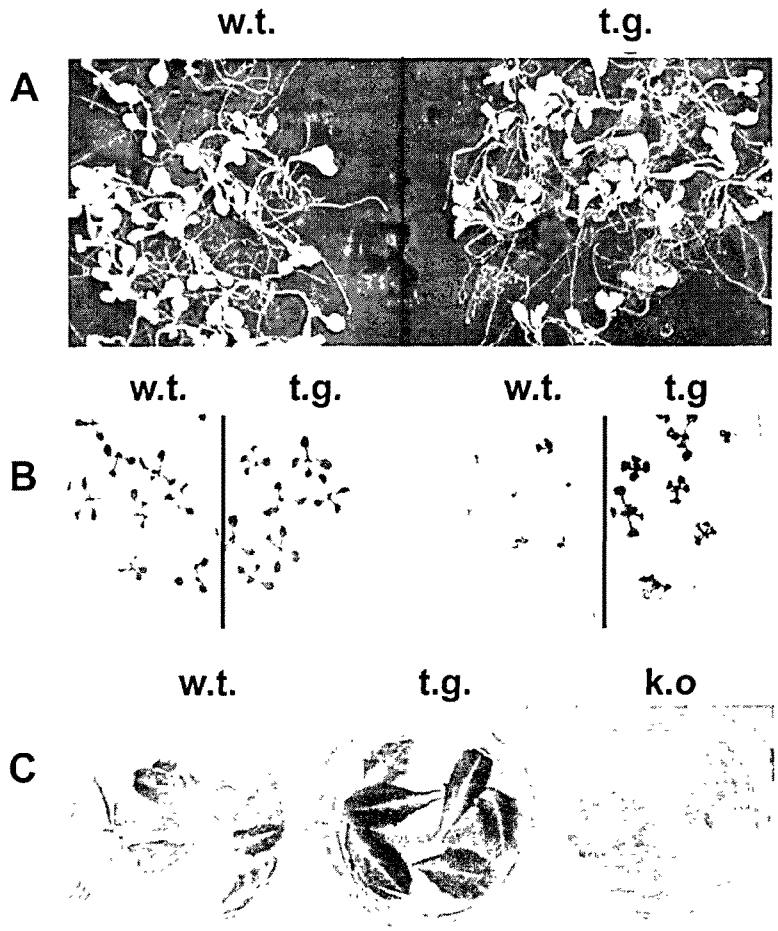


Fig. 4. *AtNDPK2*-overexpressing transgenic plants are protected from multiple stress. The environmental stress tolerance of wildtype (w.t.), *AtNDPK2*-overexpressing transgenic (t.g.) and *AtNDPK2*-knockout mutant (k.o.) plants were investigated. (A) Tolerance to cold stress. *Arabidopsis* plants were frozen at -7 °C for one hour, returned to the original growth conditions (Kim et al., 2001) and photographed a week later. The surviving plants all showed green pigmentation. (B) Tolerance to salt stress. *Arabidopsis* seedlings were raised for 2 weeks on MS medium (left panel) or 3 weeks in MS medium containing 50 mM NaCl (right panel) to assess their survival under salt stress. (C) Tolerance to MV. Fully expanded leaves from 3-week-old plants were transferred to MS liquid medium containing 1.0 μM MV for 7 days and the percentage of plants that survived wasere recorded. The Ddying plants were albino and and displayed a loss of lost chlorophyll content.