Running Title: NUDT7 modulates the basal defense response

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AtNUDT7, a negative regulator of basal immunity in Arabidopsis, modulates two distinct defense response pathways and is involved in maintaining redox homeostasis.

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ABSTRACT

Plants have evolved complicated regulatory systems to control immune responses. Both positive and negative signaling pathways interplay to coordinate development of a resistance response with the appropriate amplitude and duration. AtNUDT7, a Nudix-domain containing protein in Arabidopsis that hydrolyzes nucleotide derivatives, was found to be a negative regulator of the basal defense response and its loss-of-function mutation results in enhanced resistance to infection by Pseudomonas syringae. The nudt7 mutation does not cause a strong constitutive disease resistance phenotype but it leads to a heightened defense response, including accelerated activation of defense-related genes that can be triggered by pathogenic and non-pathogenic microorganisms. The nudt7 mutation enhances two distinct defense response pathways: one independent of and the other dependent on NPR1 and salicylic acid (SA) accumulation. In-vitro enzymatic assays revealed that ADP-ribose (ADPR) and NADH are preferred substrates of NUDT7, and the hydrolysis activity of NUDT7 is essential for its biological function and is sensitive to inhibition by Ca++. Further analyses indicate that ADPR is not likely the physiological substrate of NUDT7. However, the nudt7 mutation leads to perturbation of cellular redox homeostasis and a higher level of NADH in pathogen-challenged leaves. The study suggests that the alteration in cellular anti-oxidant status caused by the nudt7 mutation primes the cells for the amplified defense response and NUDT7 functions to modulate the defense response to prevent excessive stimulation.
INTRODUCTION

Recognition of a potentially pathogenic microorganism by plant cells triggers coordinated disease resistance responses, leading to the deployment of appropriate defense mechanisms. A strong form of disease resistance is mediated by race-specific gene-for-gene interaction in which the host Resistance (R) protein directly or indirectly recognizes the cognate Avirulence (Avr) protein delivered into the host by a specific pathogen strain (Dangl and Jones, 2001). Such an interaction activates a hypersensitive response (HR) culminating in rapid cell death of the infected site, leading to a halt of pathogen growth. Several signaling molecules/second messengers such as reactive oxygen/nitrogen species (ROS/RNS), salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) play important roles in relaying, amplifying and integrating the initial pathogen signal through complex signaling networks to activate defense mechanisms (Dangl and Jones, 2001; Hammond-Kosack and Parker, 2003). Phospholipid derivatives such as phosphoinositols and nucleotide derivatives such as cyclic GMP (cGMP) and cyclic ADP-ribose (cADPR) are also involved in the defense signaling pathways (Durner et al., 1998; Laxalt and Munnik, 2002).

In the absence of the R-Avr interactions, host cells are still capable of activating the basal defense response (Glazebrook et al., 1997; Belkhadir et al., 2004). The basal defense response can be rapidly induced upon recognition of molecular signatures common to a whole class of microorganisms (whether pathogenic or not) which are termed pathogen/microbe-associated molecular patterns (PAMPs/MAMPs) or general elicitors (Gomez-Gomez and Boller, 2002; Eulgem, 2005). The basal defense response, together with constitutive physical and chemical barriers, successfully prevents most infections from becoming established. Pathogens have evolved Avr proteins as virulence effectors to suppress the basal defense response, whereas R proteins have been evolved to detect action of effectors and to amplify the basal defense response (Jones and Dangl, 2006). Because activation of the induced defense response will likely cause deleterious effects on normal cell functioning, delicate regulation of the immune response by both positive and negative pathways is essential for an organism’s functioning (Alexander and
Hilton, 2004). However, regulatory systems that modulate the defense response in plants remain largely unknown.

Nudix box-containing proteins generally hydrolyze a nucleoside diphosphate linked to some other moiety $X$, and the proteins containing the domain were termed Nudix proteins (Bessman et al., 1996). Substrates of Nudix hydrolases include dinucleoside polyphosphates, nucleotide sugars, phosphoinositol derivatives, and capped mRNA. Some of these molecules play regulatory roles, whereas others are coenzymes and mutagenic or toxic components. It has been suggested that many Nudix hydrolases function as cellular surveillance enzymes to maintain physiological homeostasis by sensing and modulating the levels of their substrates (Xu et al., 2001). The first plant Nudix protein was isolated from *Lupinus angustifolius* as a diadenosine tetraphosphate (Ap4A) hydrolase (Maksel et al., 1998). The *Arabidopsis* genome encodes 24 members of the Nudix family (named AtNUDT) (Ogawa et al., 2005). One of the Nudix proteins, At1g68760, was found to remove pyrophosphate from dihydroneopterin triphosphate, a step in the folate synthesis pathway (Klaus et al., 2005). In-vitro enzymatic analyses on substrate specificity have been carried out for nine of the AtNUDTs (abbreviated as NUDTs here) that are predicted to be cytosolic (Ogawa et al., 2005). Biological functions of plant Nudix proteins are little known. NUDT7 was recently identified as a multiple-stress induced gene and its knockout mutant was found to show constitutive resistance against *Pseudomonas syringae* (Jambunathan and Mahalingam, 2006).

Furthermore, the *NUDT7* gene was identified as one of the pathogen-responsive genes whose induction is dependent on *EDS1* and *PAD4* (Bartsch et al., 2006). The authors also showed that the *nudt7* mutant exhibited enhanced resistance to *Hyaloperonospora parasitica* and the enhanced disease resistance phenotype requires functional *EDS1*. These results indicate that NUDT7 plays an important role as a repressor in the disease resistance pathways. However, the mechanism by which NUDT7 regulates these pathways is little understood. Interestingly, Nudix proteins have been identified as putative TTSS effectors from phytopathogenic bacteria (Mukaihara et al., 2004; Tamura et al., 2005; Koebnik et al., 2006), suggesting that pathogens may deliver Nudix protein to promote pathogenesis presumably by modulating the levels of nucleotide derivatives in host cells.
In this report, we describe the identification and characterization of NUDT7 as a negative regulator of the basal defense response. Its loss-of-function mutation leads to acceleration and amplification of the basal defense response. Two distinct pathways, one dependent on and the other independent of NPR1 and SA, are modulated by NUDT7 to prevent an excessive defense response. Our study indicates that alteration in redox homeostasis caused by the nudt7 mutation may lead to the excessive defense response that can be triggered not only by pathogens but also by other inciting agents.

RESULTS

Identification of nudt7 as an Enhanced Disease Resistance Mutant

The basal defense response can be quickly triggered upon pathogen invasion through recognition of MAMPs by host cells. It is plausible that many genes that are rapidly induced following pathogen infection may play important roles in the basal defense response. Based on gene expression profiles from our Arabidopsis Genechip microarray experiment (data not shown), we selected some early pathogen-responsive genes (induced within 1.5 hours of infection by the bacterial pathogen P. syringae) and obtained their T-DNA insertion mutagenized lines for further analyses to determine their roles in the basal defense response. Among the T-DNA insertion lines, the nudt7 mutant was found to have an enhanced disease resistance phenotype against the virulent P. syringae pv. tomato (Pst) strain DC3000. The nudt7 mutant line (Salk_046441) carries a T-DNA insertion at the locus At4g12720, which encodes a protein containing a Nudix box (see below). The gene was previously named AtNUDT7 (abbreviated here as NUDT7), one of 24 putative Arabidopsis genes encoding Nudix proteins, and was reported to possess a hydrolase activity in an in-vitro enzymatic assay (Ogawa et al., 2005). Growth of Pst in nudt7 was 3-9 fold lower than in wt plants in several independent experiments using the in-planta bacterial growth assay. Fig. 1A shows the result of one of the experiments. In addition, growth of the avirulent strain Pst avrRpm1 was also reduced in the mutant by several folds (Fig. 1B). Heterozygous
nudt7/+ plants were indistinguishable from wt plants in resistance to Pst (data not shown), indicating that the nudt7 mutation is recessive.

The morphological phenotype of the nudt7 plants differs under different growth environments. When the plants were grown in our communal growth rooms where pathogens (mainly Erysiphe cichoracearum) and insects (mainly aphids) were frequently present and pesticides were routinely used, the nudt7 plants grew much smaller than the wild-type plants (data not shown). However in isolated growth chambers where no obvious damage by pathogens or insects was observed and no pesticide or other chemical (except fertilizer) was applied, the nudt7 plants were often morphologically indistinguishable from wt plants (Fig. 1C) and were slightly smaller than the wt plants in some batches. All experimental data presented here were obtained using plants growing in the isolated growth chamber on soil unless otherwise specifically indicated in the text. In two recent reports, the same nudt7 mutant was described as having a stunting growth phenotype (Bartsch et al., 2006; Jambunathan and Mahalingam, 2006). Our results indicated that the growth stunting is likely indirectly caused by the nudt7 mutation due to the mutant’s super-sensitivity to some stimuli in growth environments. Indeed, excessive oxidative stresses could cause growth stunting of the mutant (see below).

NUDT7 consists of eight exons and the mutant has a T-DNA insertion in its first exon at 117 bp 3’ to the initial codon. A NUDT7 genomic clone (NUDT7t) including the 2-kb promoter region was obtained by PCR from genomic DNA of wt plants. When transformed into the nudt7 mutant, it was able to complement the enhanced disease resistance phenotype of the mutant (Fig. 1A), confirming that the mutation is indeed caused by the insertion at the NUDT7 gene.

NUDT7 is expressed at a low level in the uninfected wt leaves (Fig. 1D). Its expression was significantly induced in response to infection by both avirulent Pst avrRpm1 and virulent Pst strains. Strong induction of NUDT7 could be detected within 0.5 hr of the inoculation. In the Pst-inoculated leaves, its transcript level dropped at 3 hrs post-inoculation following the initial increase and then increased again at the later time points (Fig. 1D). This expression profile was repeatedly observed in multiple experiments. NUDT7 was also induced systemically by both the virulent Pst and
avirulent \textit{Pst} \textit{avrRpm1} strains (Fig. 1D). \textit{NUDT7} transcripts were not detectable in the mutant (Fig. 1E), indicating that the insertion likely results in a null allele.

The \textit{nudt7} Mutation Results in Hyper-activation of the Defense Response

To understand the mechanism by which the \textit{nudt7} mutation results in enhanced disease resistance, we used the RNA blotting analysis to reveal differences in activation of the defense response between the mutant and wt plants by examining pathogen-induced expression patterns of several pathogen-induced genes including \textit{PATHOGENESIS-RELATED (PR)} genes (\textit{PR1}, \textit{PR2}) and \textit{avrRpt2-induced gene 1 (AIG1)} (Reuber andAusubel, 1996). The mutant did not accumulate high levels of \textit{PR1}, \textit{PR2}, or \textit{AIG1} in the absence of pathogen infection; however, induction of these genes was accelerated and amplified in the mutant following pathogen inoculation (Fig. 2A-2D and data not shown). In wt plants, transcripts of these genes were detectable only after several hours post-inoculation; whereas in the mutant plants, induction of these genes became apparent in 1-2 hrs post-inoculation. Hyper-activation of the pathogen-responsive genes in the mutant can be induced not only by the avirulent \textit{Pst} strain (Fig. 2A) but also by the virulent \textit{Pst} (Fig. 2B, 2D) as well as by the \textit{Pst} mutant \textit{hrcC} and a non-host pathogen \textit{P. syringae pv. phaseolicola (Psp)} (Fig. 2C, 2D). \textit{Pst hrcC} carries a deletion in the \textit{hrcC} gene that encodes for a component of the Type III secretion system (Boch et al., 2002) and is defective in delivering Type III virulence effectors into host cells and therefore is unable to grow or cause disease in plant tissues. The RNA blotting analysis also revealed that the \textit{nudt7} mutant carrying the wild-type \textit{NUDT7} transgene no longer showed hyper-activation of the defense-related genes (Fig. 2E), again demonstrating that the \textit{nudt7} mutation is responsible for the hyper-responsiveness phenotype.

The above results indicate that the hyper-responsiveness of the \textit{nudt7} mutant is likely triggered upon the initial recognition of the microorganisms, presumably through detection of MAMPs. Indeed, infiltration of the mutant leaves with boiled \textit{Pst} and individual MAMPs such as chitosan (100µg/ml) (a main cell wall component of fungi) and flg22 (5µM) (the 22 amino acid peptide from bacterial flagellin that triggers the
innate immune response) resulted in quick and strong induction of *AIG1* and *PR2* in the mutant (Fig. 2F and data not shown).

**The nudt7 Mutation Does Not Affect Hypersensitive Cell Death**

The *nudt7* mutant does not develop spontaneous lesions visible by the naked eye. When examined under microscopes after Trypan Blue staining, the leaves of uninfected mutant plants were found to display very small lesions, most of which appear to consist of a single cell (Fig. 3A). The estimated number of the micro-lesions in expanded leaves from the mutant was approximately 150/leaf, whereas leaves from a wt plant generally displayed fewer than 20 stained spots/leaf (Fig. 3B). Diaminobenzidine (DAB) staining (to detect *H₂O₂* *in situ*) revealed no DAB positive spots in the uninfected leaves of the mutant or wt plants (data not shown), indicating that the micro-cell death of the mutant is not associated with a high level of *H₂O₂* production. Similarly, we did not detect significant difference between wt and mutant leaves in levels of H₂O₂ (Fig. 3G) determined by a quantitative hydrogen peroxide measurement method.

The micro-lesions associated with the mutant plants may be a consequence of disruption of the host cells’ normal function due to their hyper-responsiveness to microorganisms and/or other stimuli in the environment. Examination of green healthy leaves from plants grown in sterile environments in culture tubes revealed no significant difference in micro-lesions between the mutant and wild-type plants (Fig. 3C and 3D): both the mutant and wild-type leaves had fewer than 10 Trypan Blue-positive spots/leaf.

To determine whether the *nudt7* mutation enhances hypersensitive cell death triggered by avirulent pathogens, we inoculated leaves with *Pst avrRpm1* at a concentration of approximately 10⁷ cfu/ml and monitored appearance of dead cells by Trypan Blue staining. No obvious difference was found between the mutant and wild-type plants in the timing of hypersensitive cell death. In both wt and mutant leaves, dead cells were observed after approximately 6 hrs post-inoculation (Fig. 3E and 3F). Tissue collapse, the visible symptom of infected leaves, appeared approximately 10 hrs post-inoculation for both mutant and wt plants (data not shown). The virulent *Pst* strain was not able to induce cell death in either wt or mutant leaves when the leaves were observed
by Trypan Blue staining in 5-36 hr intervals after the inoculation (data not shown). We further monitored electrolyte leakage to provide a quantitative measurement of cell death in leaf discs infiltrated with Pst avrRpm1 and Pst (10^7 cfu/ml). Compared with the Pst-inoculated leaf discs, Pst avrRpm1 caused significant iron leakage, which began after approximately 2 hrs post-inoculation (Fig. 3H). However, there is no significant difference in electrolyte leakage between the infected leaf discs of the wt and nudt7 mutant plants. Besides, no difference in hypersensitive cell death was seen between the mutant and wt plants when infection was carried out with a low concentration of the bacteria (2x10^5 cfu/ml) (data not shown).

The Hyper-activation of the Defense Response in the nudt7 Mutant Is Not Primed by an Elevated Level of Salicylic Acid

Many enhanced disease resistance mutants constitutively accumulate high levels of SA. To investigate whether the hyper-responsiveness phenotype of nudt7 is caused by higher levels of SA prior and post-inoculation, we measured SA and its glucoside conjugate (SAG, the storage form of SA). As shown in Fig. 4, the mutant accumulated significantly higher levels of SA and SAG 8 hrs post-inoculation with Pst. However, within 3 hrs post-inoculation, although the mutant had slightly higher levels of SAG, there was little difference in the levels of SA between the mutant and wt plants. The results indicate that the rapid activation of the defense-related genes in the mutant is unlikely due to an elevated level of SA although the high level of SA at the later stages likely contributes to the enhanced disease resistance phenotype of the mutant.

The nudt7 Mutation Enhances Two Distinct Defense Response Pathways

The nudt7 mutant was crossed with previously identified disease resistance mutants, npr1 and eds5, and with the NahG plant to generate double mutants. NPR1 is a key component required for disease resistance (Cao et al., 1994), the eds5 mutant is defective in the SA biosynthetic pathway (Nawrath et al., 2002), and the NahG plants over-express a bacterial hydroxylase which converts SA to catechol (Delaney et al., 1994). Catechol could also cause susceptibility to non-host pathogens (van Wees and Glazebrook, 2003). These double mutants are slightly smaller than the single mutants...
Analyses of expression profiles of the defense-related genes using Northern blotting (Fig. 5B) revealed that like the nudt7 single mutant, both the nudt7 npr1 and the nudt7 eds5 double mutants exhibited pathogen-induced hyper-activation of PR2 and AIG1 genes although eds5 nudt7 showed slightly attenuated AIG1 induction. On the other hand, the npr1 and eds5 mutations not only blocked hyper-activation of PRI associated with the nudt7 mutation but also suppressed normal induction of the PRI gene by the pathogen infection. In the nudt7 NahG plants, induction of both AIG1 and PR2 was suppressed and PRI expression was abolished (Fig. 5B). Further analyses of the disease resistance phenotypes of the double mutants using the in-planta bacterial growth assays (Fig. 5C) showed that nudt7 npr1 and nudt7 eds5 double mutants were slightly more susceptible than wt plants but were more resistant than the eds5 and npr1 single mutants. The nudt7 NahG plants supported significantly more bacterial growth than wt plants although they are still more resistant than the NahG plants. These results indicate that NUDT7 modulates two distinct branches of the defense response pathways: one independent of NPR1 and SA and the other dependent on both of the two components.

**ADPR and NADH are Preferred In-vitro Substrates of NUDT7**

NUDT7 encodes a 32-kDa polypeptide that contains the conserved sequence motif called the Nudix box which is involved in hydrolyzing a nucleoside diphosphate linked to some other moiety X (Bessman et al., 1996). The Nudix motif consists of the highly conserved consensus sequence GX₅EX₇REUXEEXGU where X can be any amino acid and U is an aliphatic hydrophobic acid. A general method for the activity assay of Nudix hydrolases (Xu et al., 2004) was used to determine enzymatic activity of NUDT7 expressed in and purified from E. coli as a fusion protein to GST (Fig. 6A). In this assay, hydrolysis of a substrate (nucleoside diphosphate-X) will generate phosphatase-sensitive products, which, in the presence of intestinal phosphatase, will release inorganic phosphate that can then be quantified colorimetrically. NUDT7 was found to be active on several known Nudix hydrolase substrates such as ADPR, NADH, NAD, NADPH, NADP, and diadenosine tetraphosphate (Ap4A) but showed essentially no activity on cyclic ADPR (cADPR), GDP-mannose, UDP-glucose, ATP, or GTP (Fig. 6B and Table
1). Besides, NUDT7 was not able to hydrolyze ADPR in poly- or mono-ADP-ribosylated polypeptides (data not shown). Among these nucleotide analogs, ADPR and NADH are NUDT7’s preferred substrates. The NUDT7’s hydrolase activity was inhibited in the presence of Ca\(^{++}\) (Fig. 6C and Table 1). Later, we used \textit{E. coli}-expressed NUDT7 without the GST tag for the activity assays on these nucleotide analogs and observed similar results (data not shown).

We generated a mutant form of NUDT7 (NUDT7 E154Q) in which the conserved glutamate residue E154 in the Nudix box is mutagenized to glutamine. E154 likely binds to Mg\(^{++}\) to carry out the nucleophilic attack and therefore is considered essential for its hydrolase activity (Mildvan et al., 2005). \textit{E. coli}-expressed E154Q was found inactive against the nucleotide derivatives such as ADPR and NADH (Fig. 6D and 6E). The E154Q mutant whose expression is under the control of the \textit{NUDT7} promoter was then transformed into the \textit{nudt7} mutant plants and was found unable to complement the \textit{nudt7} mutant phenotype. Like \textit{nudt7}, the \textit{nudt7} E154Q plants still showed hyper-activation of \textit{PR1}, \textit{PR2}, and \textit{AIG1} (Fig. 6F) and enhanced resistance to \textit{Pst} (data not shown), demonstrating that NUDT7’s hydrolase activity is required for its normal biological function although it is possible that the E154Q mutation may cause another unknown effect on the protein function in addition to abolishing its hydrolase activity.

The \textit{nudt7} Mutation Affects Redox Homeostasis

The in-vitro hydrolase activity assays described above raise the possibility that ADPR could be the physiological substrate of NUDT7. We reasoned that if NUDT7 functions to modulate the ADPR level, the \textit{nudt7} mutation is expected to lead to an increase in the level of ADPR. To test the working hypothesis, we determined levels of free ADPR. Free ADPR levels in \textit{Arabidopsis} leaves were found to be too low to be detected in our initial nucleotide profiling analyses using regular high-performance liquid chromatography (HPLC) (data not shown). We then applied a HPLC technique for detection of ethano-ADPR, a fluorescence analog of ADPR for measuring ADPR (see Materials and Methods). Our results showed that the concentration of ADPR in \textit{Arabidopsis} leaves is around 200 pmol/g fresh weight (FW) (Fig. 7A), which will give a
rough estimate of about 20-40 uM in cellular compartments (such as the cytosol) according to an estimation method assuming that amount of ADPR is inappreciable in the vacuole (Noctor, 2006). We did not detect significant difference in the ADPR levels between the mutant and wt leaves or between pathogen-infected and uninfected leaves. These results argued against ADPR being a physiological substrate of NUDT7. This notion is further supported by the result of our enzyme kinetic assay which showed that Km for the hydrolysis of ADPR by NUDT7 is 0.602±0.056 mM, apparently too high for APDR to be an in-vivo substrate.

The result from the in-vitro enzymatic assays also suggested that NUDT7 could be a redox modulator since it hydrolyzes NADH and NADPH. We determined levels of NAD(P) and NAD(P)H in wt and nudt7 leaves before and after infection with P. syringae (Fig. 7A). Concentration of each of these nucleotide analogues in leaves ranged from 30-80 nmol/g tissue (roughly several mM to 20 mM in cellular compartments excluding the vacuole based on the estimation method mentioned earlier). Km for the hydrolysis reaction of NADH by NUDT7 was found to be 0.1281±0.0155mM. As shown in Fig. 7A, the levels of NADPH and NADP did not change much before and after pathogen incubation. However, slightly more NADPH was detected in the nudt7 mutant at 6 hrs post-infection (the difference is not statistically significant). The levels of both NADH and NAD significantly increased in the mutant and wt leaves at 6 hrs post-infection. At that time point, the concentration of NADH in the mutant leaves was significantly higher than that in the wt leaves.

To get more information on overall cellular redox states, we further determined levels of GSH and GSSG, an important antioxidant buffer system involved in scavenging attacking radicals as well as in regenerating oxidized biomolecules. In both wt and mutant leaves, GSH/GSSG ratios decreased gradually following the pathogen infection, reflecting an increasing oxidative stress (Fig. 7B). However, the ratios in the mutant were consistently lower than those in wt in both uninfected and infected leaves. However, the mutant leaves contained a consistently higher level of total glutathione. These results indicate that the nudt7 mutation leads to an increase in oxidation of cellular components which in turn promotes cells to increase production of total glutathione and NAD(P)H to counteract the perturbation of cellular redox balance. To determine whether the escalated
oxidative state associated with the mutant makes it more sensitive to extra oxidative stresses, we examined tolerance of plants to application of Paraquat (PQ), a ROS-generating chemical. As shown in Fig. 8, the mutant plants were more sensitive to the externally applied oxidative stress. Leaves of the mutant plants, especially older leaves, became wilted within 24 hrs after spraying with 10um PQ (Fig. 8A) and showed clear necrotic lesions several days later (Fig. 8B) while the effect on the wt plants was very mild. The nudt7 eds5 (Fig. 8A), nudt7 npr1 (Fig. 8B), and nudt7 NahG (data not shown) plants were as sensitive as the nudt7 single mutant to the PQ treatments. Multiple applications of a lower concentration of PQ starting at a younger seedling stage caused striking growth stunting to the mutant plants (Fig. 8C).

**DISCUSSION**

Basal immunity is apparently controlled by complex pathways. Many important genes that control the basal defense response have likely eluded identification through classical forward genetic approaches because of the complexity of the pathways and functional redundancy. We have taken the reverse/functional genomic approach to identify components involved in basal immunity. Loss-of-function of NUDT7 was found to enhance resistance against *P. syringae*, indicating that it acts as a negative regulator of disease resistance. The mutant does not display constitutive accumulation of high levels of *PR* gene transcripts in absence of pathogen infection; however, the mutation potentiates a remarkably stronger defense response that can be triggered by both pathogenic and non-pathogenic bacterial strains.

In the two previous reports by other groups characterizing the same mutant line, the mutant plants were found to have a stunting growth phenotype and showed constitutive accumulation of very high levels of *PR* gene transcripts and SA (Bartsch et al., 2006; Jambunathan and Mahalingam, 2006). Our study reveals that the *nudt7* mutation does not directly cause such a strong constitutive disease resistance phenotype. Instead, the mutation causes the plants to be ‘primed’ for an accelerated defense response upon recognition of an inciting agent. Besides pathogen infection, other inciting agents can also trigger activation of the abnormally regulated defense response in the mutant.
These stimuli may include non-pathogenic microorganisms in growth environments or some chemicals.

Innate immunity is considered a double-edged sword. Delicate regulation of the innate immune response is not only important for hosts to defend invading pathogens but also essential for co-habiting with non-pathogenic microorganisms in the environment (Kobayashi and Flavell, 2004). NUDT7 likely acts as a component in a negative feedback loop of the basal defense response to prevent unnecessary cell stimulation. The analyses of the nudt7 mutation coupled with the other disease resistance mutants indicate that NUDT7 modulates two defense response pathways. The nudt7 mutation enhances one branch of the pathway that leads to activation of a subset of pathogen-induced genes including PR1. This branch of the pathway is dependent on both NPR1 and SA. The mutation also enhances another branch of the defense response that activates expression of another subset of pathogen-induced genes such as PR2 and AIG1, which is largely independent of NPR1 or SA. It has been reported that neither NPR1 or SA was required for flagellin-induced innate immunity (Zipfel et al., 2004). Because the nudt7 mutant also exhibited hyper-activation of the defense-related genes triggered by the MAMPs including flagellin and chitosan, NUDT7 likely functions as a modulator of the innate immune response.

NUDT7 may play its role by sensing and modulating the levels of its substrate(s) to fine-tune the defense response. Identification of ADPR and NADH as its preferred in-vitro substrates raises the possibility that these nucleotide analogs may be physiological substrates of NUDT7 and act as regulatory molecules in plant disease resistance signaling. A similar finding led Jambunathan and Mahalingam (2006) to propose that ADPR and NADH are in-planta substrates of NUDT7. However, our results suggest that ADPR is unlikely the physiological substrate of NUDT7 since the mutant does not accumulate a higher level of ADPR. Neither did we detect a significant change of the level of ADPR in response to pathogen infection. Although Jambunathan and Mahalingam (2006) reported that untreated nudt7 plants accumulated over two fold more NADH than wt plants, we did not find any significant difference in the levels of NADH in untreated mutant and wt leaves. Besides, the levels of NADH in wt plants determined by Jambunathan and Mahalingam (2006) are about several hundred-fold higher than
those from our results and from other reports (Noctor, 2006). It is not clear whether the
difference is caused by different growth conditions. However, we did find that the
NADH level in mutant leaves at 6 hrs post-infection was approximately 40% higher than
that in wt plants. Although the results suggest that NADH could be a biologically
important substrate of NUDT7, we could not rule out the possibility that the higher level
of NADH in the mutant may be indirectly caused by the mutation. The nudt7 mutation
leads to an escalated oxidative stress, as indicated by having a lower GSH/GSSG ratio
than wt plants in both infected and untreated leaves, which could cause cells to produce
more NADH as an attempt to maintain the antioxidative power of glutathione. Another
indication that NADH may not be the physiological substrate is that we did not find any
significant difference in the levels of NADH between uninfected leaves of wt and mutant
plants. In addition, NUDT7 over-expression lines did not differ from wt plants in the
NADH levels and neither have we observed any difference in disease resistance or
defense-related gene induction between wt and the over-expression lines (data not
shown).

A more direct effect caused by the nudt7 mutation was found to be the
perturbation of cellular redox homeostasis. Unlike the levels of NADH or SA which
were not different between the untreated wt and mutant plants, the ratios of GSH/GSSG
were consistently lower in the mutant, suggesting that more GSH molecules were used to
regenerate oxidized biomolecules. Although the precise mechanism by which the nudt7
mutation causes the change in the glutathione status remains to be determined, the
perturbation in redox balance may ‘prime’ cells to hyper-respond to inciting agents such
as pathogen infection, resulting in an excessive defense response and enhanced disease
resistance. It is believed that anti-oxidant status sets the threshold for general defense
responses provoked by biotic or other stresses (Foyer and Noctor, 2005).

It is worth mentioning that primary biochemical functions of a Nudix protein may
not always be a hydrolysis of its substrate. Instead, a Nudix box may be involved in
conformational change of a Nudix-containing protein through its binding to a nucleotide
analog. In animal immunocytes, the Ca++-permeable cation channel LTRP2 mediates
Ca++ influx to trigger the immune responses (Perraud et al., 2001; Hara et al., 2002).
LTRP2 contains a Nudix box at its C-terminal portion. ADPR regulates the channel’s
activity by binding to the Nudix box which also acts as an intrinsic deactivation mechanism for LTRP2 through its ability to break down ADPR. Interestingly, a Nudix protein from cauliflower was found to be an inhibitor of glutamine synthetase and nitrate reductase, and the inhibition is dependent on NADH but relieved by ATP and AMP (Moorhead et al., 2003). The mechanism of the inhibition and its biological significance is not known. Recently, it was shown that Arabidopsis DCP2, a Nudix protein, interacts with DCP1 and VCS to form a mRNA decapping complex (Xu et al., 2006). It is interesting to explore whether NUDT7 regulates activity of other proteins through protein-protein interaction mediated by binding of nucleotide analog to its Nudix domain.

The Arabidopsis genome encodes over 20 members of the Nudix family. Their biological functions and their modes of action remain largely unknown. It is plausible that biological substrates of some AtNUDT proteins are nucleotide analogs. A variety of nucleotide derivatives, including ADPR, cGMP, cADPR, AP4A and cytokinins, play regulatory roles in many biological processes. Nudix hydrolases may be involved in biological processes by sensing and modulating levels of nucleotide analogs. The identification of the Nudix proteins as putative virulence effectors of phytopathogens (Mukaihara et al., 2004; Tamura et al., 2005; Koebnik et al., 2006) suggest that pathogens may use Nudix proteins to mimic a host’s cellular functions. IalA, a Nudix protein encoded by one of two invasion-associated loci of Bartonella bacilliformis that is the only bacterium that infects human red blood cells, is also a virulence factor essential for infectivity (Mitchell and Minnick, 1995; Conyers and Bessman, 1999). When expressed in E. coli, IalA can markedly increase E. coli’s capacity to invade red blood cells (Mitchell and Minnick, 1995). Definitive understanding of how Nudix proteins such as NUDT7 are involved in modulating the host defense response will provide novel and important insights into the molecular mechanisms of disease resistance in plants.

MATERIALS AND METHODS

Plant Growth
Plants were grown at 22°C with 50% humidity and under short day conditions (9/15 hr photoperiod at a light intensity of 125 mol m² s⁻¹).

**PCR Analysis of the nudt7 Mutant Allele**

The T-DNA-flanking genomic fragment was amplified by PCR from Salk_046441 using the gene specific primer 4g12720p5 (5’-CAACGAGATGAGTCCAATAAACAAA-3’) and the T-DNA left border primer LBb1 (AGTTGCAGCAAGCGGTCCACGC). The wt fragment of this region was amplified using the primer pair 4g12720p5 and 4g12720p3 (CTAAACAATACGCTGACACCCTTCA).

**Nucleic Acid Analysis**

*Arabidopsis* genomic DNA was isolated from leaves using a modified CTAB procedure (Saghai-Maroof et al., 1984). Total RNA was isolated from leaf tissues using the Trizol (Promega, Madison, WI) method for Northern or the on-column DNase digestion RNA preparation columns (Sigma, St. Louis, MO) for Q-PCR analysis. Nucleic acid electrophoresis, transferring onto nylon membranes, ³²P labeling of DNA probes (27-9240-01, Amersham Biosciences, Piscataway, NJ), and hybridization were performed according to the standard protocols (Sambrook et al., 1989).

**Plant Pathogens and Inoculation**

*P. syringae* was grown as described (Cameron et al., 1994). *P. syringae pv. phaseolicola* was provided by Dr. Jian-Min Zhou and all other strains were from Dr. Barbara Kunkel. To prepare inoculated leaf tissues for RNA preparation, leaves were infiltrated with 1x10⁷ cfu/ml bacterial suspension (in water) with a syringe. For the in planta bacterial growth assays, 3x10⁴ cfu/ml (for virulent strains) or 1x10⁵ cfu/ml (for avirulent strains) bacterial suspension was infiltrated into leaves. Eight leaf discs were
collected per replicate and each data point represents three replicates. All bacterial growth assays were repeated, and only results that were observed consistently are shown.

**Treatments with MAMPs**

To prepare boiled *Pst* and *E. coli*, the bacterial suspensions (10^7 cfu/ml in ddH2O) were boiled for 10 minutes and cooled before being infiltrated into leaves. flg22 (QRLSTGSRINSKDDAAGLQIA) was synthesized by GenScript Corporation (Piscataway, NJ). Chitosan (Sigma, 52368) and lipopolysaccharide (Sigma, 62326) were all purchased from Sigma (St. Louis, MO). Induction of the defense response was conducted by pressure infiltration of the MAMP solutions into Arabidopsis leaves using 1ml syringes (approximately 20µl/leaf).

**Complementation of the nudt7 Mutant**

A 4.2 Kb fragment of *NUDT7* genomic DNA (*NUDT7*), which includes a 2 kb promoter region and 0.4 kb 3’ UTR, was amplified from Col-0 by using the primer pair 4g12720p5Kpn (GGATCCGGAGCTAAGCATCTGAATCAG) and 4g12720p3Sal (GTCGACCAGTGTAGTAAATGGTCAAGAGAC). The PCR product was cloned into pCR-Blunt II-TOPO (Invitrogen, CA), sub-cloned into the binary vector pPZP221 using the restriction sites KpnI and Sal I, and then transformed into the mutant plants.

**Measurement of SA and SA Glucoside**

SA and SAG were extracted and analyzed as previously described (Uknes et al., 1993). Reverse-phase HPLC on a C-18 column (ODS-AM analytical column from Waters Corp, Milford, MA) was used in the analysis.

**DAB Staining, Trypan Blue Staining, H₂O₂ Measurement, Electrolyte Leakage Analysis**
DAB staining and Trypan Blue staining were carried out as previously described (Alvarez et al., 1998). Determination of H2O2 levels was carried out using the modified chromogenic peroxidase-coupled assay (Veljovic-Jovanovic et al., 2002). The electrolyte leakage assay was performed according to the previously described method (Torres et al., 2002). Briefly, leaves were infiltrated with bacterial suspensions at 10^7 cfu/ml in ddH2O. Leaf discs (0.25cm² each) were collected from the inoculated areas 15 minutes post-inoculation and washed 4 times with ddH2O for about 15 minutes. Six leaf discs were placed in a test tube with 4ml of ddH2O. Each treatment was replicated three times. Conductivity was measured over time using an ORKTON pH/conductivity meter (510 series).

NUDT7 Expression in E. coli and Activity Assays

NUDT7 cDNA was cloned through RT-PCR from a RNA sample extracted from leaves inoculated with P. syringae. After reverse transcription with oligo dT as a primer, NUDT7 cDNA was amplified using the primer set TAGGATCCGGTACTAGAGCTCAGCAG and CAGTCGACTCAGAGAGAAGCAGAGGC. The 0.8 kb PCR product was cloned into pCR-Blunt II-TOPO and the cDNA insert was then dropped by digesting with BamHI/HindIII and ligated into pET41a (Novagen) to generate a fusion protein with GST at its N-terminus. The fusion construct was transformed into DL21(DE3). After the bacterial culture grew to OD600=0.6~0.8, 1 mM IPTG was added to the culture which was then grown at 30°C for additional 4 hrs. Purification of GST-NUDT7 was performed using the Glutathione-Agarose affinity resin (Sigma, G4510) according to the manufacture’s instructions.

A general method for the activity assay of Nudix hydrolases was used to determine enzymatic activity of NUDT7 (Xu et al., 2004). The assays were carried out in a 50 µl hydrolysis mixture of 50 mM Tris-HCl pH8.5, 2 µg GST-NUDT7, 5 mM MgCl2, 1 mM DTT, 2 mM substrate, and 4 units of alkaline phosphatase. After incubation for 30 min at 37°C, the reaction was stopped by adding 150 µl 1N HCl, followed by adding 100 µl H2O and 700 µl of a mixture which was freshly made by mixing 0.42%
molybdate.4H2O with 10% ascorbic acid (6:1). The reaction tubes were put in 45°C water bath for 20 min for color development and then cooled down to room temperature. The solutions were measured using a spectrophotometer at A820. The reaction without GST-NUDT7 was used as a blank control for each substrate. As for nucleoside triphosphate substrates ATP and GTP, alkaline phosphatase was replaced by inorganic pyrophosphatase and the substrates were removed by charcoal before color development. CaCl2 was added to the hydrolysis mixture to a concentration of 5 mM when investigating effects of Ca++ on NUDT7 activity. All the substrates were purchased from Sigma.

For enzyme kinetic assays, the reaction was stopped by adding 1N HCl at different time points. Concentrations of the substrates used in the kinetic assay were 0.5mM, 2mM, 5mM, 10mM, and 20mM. The kinetic assay for each substrate concentration was repeated at least three times. Km and Vmax were calculated using the Lineweaver-Burk plot based on Michaelis-Menton equation.

Site-directed Mutagenesis

The NUDT7 genomic clone used for the complementation experiment and the NUDT7 cDNA clone used for expression in E. coli were mutagenized using QuikChange XL Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacture’s instruction manual. E154Q mutation was generated by using the primer pair E154Qf (GATATATGGGACTGGAGTAGCTAGCAAGTGGAAGAAGAAACTGG) and E154Qr (CCAGTTTCTTCTCTCCACTTGCCCTAGCTACTCCAGTCCATATATC). The underlined nucleotides indicate the position of mutagenesis. The mutagenized genomic clone was then sub-cloned into the binary vector pPZP221 and transformed into nudt7 mutant plants. The mutagenized cDNA clone was sub-cloned into the E. coli expression vector. Expression and purification of NUDT7 E154Q followed the same protocol as that for wild-type NUDT7.

Determination of Glutathione, ADPR, NAD(P), and NAD(P)H
Glutathione levels were determined according to previously described method (Griffith, 1980). Plant tissues were extracted and deproteinated with metaphosphoric acid. Total glutathione is determined based on a recycling enzymatic reaction in which GSH is oxidized by DTNB to form the 412 nm chromophore TNB and another product GS-TNB, which can be reduced to GSH again by glutathione reductase (GR) in the presence of NADPH. The rate of TNB formation was monitored at 412 nm and the glutathione concentration was evaluated by comparison with a standard calibration curve. For determination of GSSG, 2-vinylpyridine is used to mask GSH.

For ADPR determination, extraction, purification and HPLC separation of cellular ADP-ribose was performed essentially as previously described (Gasser and Guse, 2005). Extracted nucleotide samples from 100 mg plant tissues were injected into the Beckman’s Gold HPLC system (Beckman, Ramsey, MN) with a YMC-ODS column (250 mm x 4.6 mm, particle size 5 µm) protected by a guard column (17 mm x 4.6 mm) (both columns were from Waters Corporation, Milford, MA). 400 µL fractions corresponding to the retention time of ADP-ribose (22.8–23.6 min) were collected. Fluorescence labeling and the HPLC detection for ethano-ADPR was carried out as described (Bobalova et al., 2002). The HPLC system and columns were the same as above but equipped with a fluorescence detector (Model FP150, JASCO Company, Japan). The fluorescence detector was set to record signals at an excitation wavelength of 230 nm and emission wavelength of 410 nm.

The pyrimidine nucleotides were measured by the cycling assay as previously described (Matsumura and Miyachi, 1980). NADH and NADPH were extracted with 0.1 M NaOH, while NAD and NADP were extracted with 0.1 M HCl. The assays involve the phenazine ethosulfate (PMS)-catalyzed reduction of Thiazoly Blue Tetrazolium Bromide (MTT) in the presence of ethanol and alcohol dehydrogenase (ADH) (for NAD and NADH) or glucose 6-phosphate and G6PDH (for NADP and NADPH). The rate of reduction of MTT was monitored at 570 nm.

Treatments of Plants with Paraquat
Paraquat (methyl viologen, Sigma 856177) was dissolved in water and sprayed onto plants using a hand spray bottle in solution containing 0.02% Silwet-77 as a detergent.

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**Figure legends**

**Figure 1.** The nudt7 mutant exhibits enhanced resistance to *P. syringae*.  
A, *In-planta* growth of *Pst* in wt (Col), nudt7, and the mutant complemented with the wt genomic clone (nudt7 NUDT7t).  
B, *In-planta* growth of *Pst avrRpm1* in wt and nudt7 plants. Each data point in A and B represents the average of three replicates ±SD.  
C, Five-week-old wt and mutant plants showing no obvious morphological difference.  
D, A Northern blotting result showing expression profiles of NUDT7 in wt plants in response to inoculation with *Pst avrRpm1* and *Pst*. The lanes labeled with 48 contain RNA samples from uninfected leaves harvested 48hrs after 4 other leaves of the same plants had been inoculated.  
E, A RNA blot showing that NUDT7 transcript was not detectable in the mutant. RNA was harvested from wt and mutant leaves 0 and 3 hrs post-inoculation with *Pst avrRpm1*.

**Figure 2.** The nudt7 mutation leads to hyper-activation of defense-related genes.  
A. Expression patterns of *PR1* in wt and mutant leaves following inoculation with *Pst avrRpm1*.  
B. Expression patterns of *PR2* induced by *Pst*. ‘Mock’ represents a RNA sample from water-inoculated leaves.  
C. Accumulation of *PR2* transcripts in wt and the mutant after inoculation with the non-host pathogen *Psp* and *Pst hrcC*.  
D. Hyper-activation of *AIG1* in the mutant in response to *Pst* or *Pst hrcC*.  
E, The mutant plants carrying the genomic clone of NUDT7 no longer exhibited hyper-activation of *PR2* (the upper panel) or *AIG1* (the lower panel) by *Pst avrRpm1*. The leaves were collected 1.5 hrs post-inoculation.  
F, Induction of *AIG1* in the nudt7 and wt plants 1.5 hrs post-infiltration with boiled *Pst*, chitosan, and flg22.

**Figure 3.** The nudt7 mutation does not enhance the hypersensitive cell death.  
A, Spontaneous micro-lesion formation in an uninfected mutant leaf observed after Trypan Blue staining.  
B, A portion of a leaf from a wt plant grown in the same environment as the mutant plant shown in (A).  
C and D, Trypan blue-stained leaves from a mutant plant (C) and a wt plant (D) grown in a petri-dish with sterile conditions.  
E and F, Hypersensitive cell death in wt (E) and nudt7 (F) leaves induced by *Pst avrRpm1*. The
leaves were taken 8 hrs post-inoculation and massive cell death (blue staining) was observed in the inoculated sites (arrows) of the both leaves. G, H$_2$O$_2$ levels in mutant and wt leaves. H, Electrolyte leakage following inoculation of wt and nudt7 leaves with Pst and Pst avrRpm1. Values are the averages of four replicates ±SD.

**Figure 4.** Levels of SA and SAG in un-inoculated and Pst-inoculated leaves of nudt7 and wt plants. Values are the averages of three replicates ±SD.

**Figure 5.** NUDT7 modulates two distinct branches of the defense response.  
A, Morphological phenotypes of the single and double mutants. B, Northern blotting results revealing effects of npr1, eds5, and NahG on pathogen-induced expression of the defense-related genes associated in the nudt7 mutant. Leaves were inoculated with Pst avrRpm1. 'M' represents mock samples harvested 1.5 hrs post-treatment with water. C, Effects of npr1, eds5, and NahG on disease resistance of the nudt7 mutant. Shown is the result of an in-planta bacterial growth assay. Bacterial numbers were counted 3 days post-inoculation with Pst. Values are the averages of three replicates ±SD.

**Figure 6.** NUDT7 is a nucleoside diphosphate hydrolase.  
A, SDS-PAGE analyses of recombinant NUDT7 purified from E. coli. The first two lanes are soluble protein extracts of the E. coli cultures without or with induction of the recombinant protein by IPTG. The protein bands were visualized with Coomassie staining. B, Hydrolase activities of NUDT7 against several nucleotides. The intensity of the blue color is directly related to the NUDT7 activity. The reaction was carried out for each substrate in the presence (+) or absence (-) of the purified NUDT7 protein. C, NUDT7 is sensitive to inhibition by Ca$^{++}$. D, SDS-PAGE analysis of purified E154Q. E, E154Q is inactive on the nucleotide derivatives. F, E154Q is unable to complement the nudt7 mutant phenotype. One and half hours post-inoculation with Pst avrRpm1, the nudt7 plants carrying the NUDT7p::E154Q construct show normal accumulation of E154Q transcripts (the upper left panel); however, they still show hyper-activation of PRI (3 hrs post-inoculation), PR2, and AIG1 (8 hrs post-inoculation).
Figure 7. The nudt7 mutation affects cellular redox homeostasis. Levels of ADPR, NAD, NADH, NADP, NADPH (A), and glutathione (B) in pathogen-challenged (infiltrated with 2x10^7 cfu/ml Pst avrRpm1) and control plants were determined. The lower left panel shows the ratios of GSH/GSSG. Values are the averages of three replicates ±SE.

Figure 8. The nudt7 mutant is super-sensitive to Paraquat. A, One day after spraying 4-week old plants with 10uM PQ, some leaves of nudt7 and nudt7 eds5 plants became wilted, whereas the wt plant showed a much mild symptom. B, Five days after the PQ treatment, some leaves of nudt7 and nudt7 npr1 plants showed the necrotic symptom. C, PQ application causes pronounced growth stunting to the nudt7 mutant. The plants were sprayed with 5uM PQ every 4 days starting at the 3-week-old seedling stage. The photo was taken 4 days after the 3rd application of PQ.
Table 1. Hydrolysis activities of NUDT7 on nucleotide derivatives

| Substrate                  | Activitya |
|----------------------------|-----------|
| ADPR                      | 43.3±5.5  |
| NADH                      | 43.7±7.7  |
| NAD                        | 12.9±2.6  |
| NADPH                     | 17.0      |
| NADP                      | 4.8       |
| FAD                       | 19.1±1.1  |
| Ap4A                      | 14.6±1.6  |
| NAADP                     | 6.9       |
| cADPR                     | 2.3       |
| GDP-mannose               | 4.0       |
| UDP-glucose               | 0.3       |
| ATP                       | 2.5       |
| GTP                       | 0.1       |
| ADPR+Ca++                 | 6.8±0.5   |

aThe rates of hydrolysis are presented as µmol/mg protein/30 minutes (± standard deviation)
(A) Bacterial growth [log(cfu/cm²)] over different days post-inoculation for Col, nudt7, and nudt7 NUDT7t.

(B) Bacterial growth [log(cfu/cm²)] over different days post-inoculation for Col and nudt7.

(C) Images showing plant growth in different conditions.

(D) Gene expression of NUDT7 under Pst avrRpm1 and Pst conditions for different time points.

(E) Gene expression of NUDT7 under different conditions with time points.
A table summarizing the expression of PR1 and PR2 genes under different conditions:

|   | wt | nudt7 | wt | nudt7 | wt | nudt7 | wt | nudt7 |
|---|----|-------|----|-------|----|-------|----|-------|
| 0 | 1  | 1     | 3  | 8     | 24 |       | 1  | 3     |
| 24 hr |     |       |     |       |     |       |     |       |

PR1 and PR2 expression levels are shown in the corresponding gel images.

A schematic showing the expression pattern of PR2 under mock and Pst treatments.

B: Mock and Pst treatments on PR2 expression.

C: Mock and Pst hrcC treatments on PR2 expression.

D: Mock and Pst treatments on AIG1 expression.

E: nudt7 and wild type (wt) treatments on PR2 and AIG1 expression.

F: Various treatments (mock, Boiled Pst, Chitosan, flg22) on AIG1 expression.
**Figure A:**
- **eds5**
- **nudt7**
- **eds5**
- **NahG**
- **nudt7**
- **NahG**
- **npr1**
- **nudt7**
- **npr1**

**Figure B:**
- **AIG1**
- **PR2**
- **PR1**

**Figure C:**
- **Bacterial number (cfu/leaf disc)**
- **Col**
- **nudt7**
- **nudt7/npr1**
- **nudt7/NahG**
- **nudt7/eds5**
- **npr1**
- **eds5**
- **NahG**

**Table:**

|        | Col | nudt7 | nudt7/npr1 | nudt7/NahG | nudt7/eds5 | nudt7/npr1 | nudt7/NahG | nudt7/eds5 | nudt7/npr1 | nudt7/NahG | nudt7/eds5 | nudt7/npr1 | nudt7/NahG | nudt7/eds5 | nudt7/npr1 | nudt7/NahG | nudt7/eds5 | nudt7/npr1 | nudt7/NahG | nudt7/eds5 | nudt7/npr1 | nudt7/NahG | nudt7/eds5 |
|--------|-----|-------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|
| M      | 1.5 | 6     | 24         | M          | 1.5        | 6          | 24         | M          | 1.5        | 6          | 24         | M          | 1.5        | 6          | 24         | M          | 1.5        | 6          | 24         | M          | 1.5        | 6          | 24         |
| hr     |     |       |            |            |            |            |            |            |            |            |            |            |            |            |            |            |            |            |            |            |            |            |            |
