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Modulation of the subcellular levels of redox cofactors by Nudix hydrolases in chloroplasts☆

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ABSTRACT

Chloroplasts are organelles that generate reactive oxygen species (ROS) through redox reactions during photosynthesis. It is now widely accepted that the redox state of the organelle is a crucial factor in determining various physiological activities in plants such as stress responsiveness and immunity. In the chloroplast, two important nucleotide cofactors, NADPH and flavin adenine dinucleotide (FAD), are well known to be indispensable for many redox reactions and therefore its normal function. The redox states and levels of NADP(H) in particular are crucial factors in ROS homeostasis in chloroplasts because of their roles as electron acceptors in photosynthetic electron transport and reducing equivalents for antioxidant systems. In contrast, FAD is the essential cofactor for enzymes involved in the biosynthesis and recycling of ascorbate, the most abundant antioxidant in plants, as well as for blue-light signaling and photosynthesis. Thus, the metabolisms of these redox cofactors must be tightly regulated. The Nudix (Nucleoside diphosphate linked to some other moiety X) hydrolases (NUDXs) are a protein family that possesses pyrophosphohydrolase activity toward a wide variety of nucleoside diphosphate derivatives including the cofactors NAD(P)H and FAD. *Arabidopsis thaliana* possesses 28 NUDXs (AtNUDX1-27 and DCP2), which are divided into three groups based on their intracellular localization (*i.e.*, cytosol, chloroplast, and mitochondrion). Of these, it has been demonstrated that AtNUDX19 regulates the intracellular levels and redox status of NADPH in the chloroplast and is involved in photosynthesis as well as stress and hormonal responses. AtNUDX23 participates in maintenance of the balance between FAD and flavin mononucleotide (FMN) levels by feedback regulation of the metabolism of flavins in the chloroplast. This review summarizes the physiological roles of NUDX enzymes as modulators of the subcellular levels of redox cofactors which can affect plant stress responses and physiological processes, and introduces recent progress in the studies of NUDXs in various organisms.

1. Introduction

Various metabolic processes in aerobic organisms directly and/or indirectly produce reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide anion (O_2–), hydroxyl radicals (HO·) and various peroxides (ROOR') and hydroperoxides (ROOH). ROS are continuously formed in compartments of the cell as an unavoidable by-product of aerobic metabolism and certain redox reactions (Mittler, 2002; König et al., 2012; Foyer and Noctor, 2013). In plants, the ROS-producing compartments are the chloroplasts, which mainly produce O_2^-, H_2O_2, and $^1O_2$ as a byproduct of photosynthesis; mitochondria, which mainly produce O_2^- and H_2O_2 as a byproduct of respiration; peroxisomes, which mainly produce H_2O_2 as a byproduct of photorespiration; and apoplasts, which mainly produce O_2^- (and subsequently H_2O_2) (Inupakutika et al., 2016). Of these, the chloroplasts are responsible for photosynthesis, and generating molecular oxygen and reducing power from the oxidation of water in the presence of light. These organelles therefore also produce significant levels of ROS (Asada, 1999; Maruta et al., 2016a). The rate of ROS production in the chloroplast can be accelerated by changes in environmental conditions, such as oxidative stress. For example, the over-reduction of the photosynthetic electron transport (PET) chain under a high light intensity results in the

Abbreviations: ABA, abscisic acid; APX, ascorbate peroxidase; FAD, flavin adenine dinucleotide; JA, jasmonic acid; KO, knock-out; NRG, NADH-responsive gene; Nudix, Nucleoside diphosphate linked to some other moiety X; NUDX, Nudix hydrolase; RF, riboflavin; ROS, reactive oxygen species; PET, photosynthetic electron transport; PAR, poly(ADP-ribosyl)ation; SA, salicylic acid

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depletion of $NADP^+$, a terminal electron acceptor, and therefore in an increase in ROS production (Asada, 1999). Under conditions of water restriction, salinity, and low temperatures, increased ROS production occurs in the PET even under normal or low light intensity. Such conditions cause the inhibition of the Calvin cycle, which require the reducing power of NADPH. This can therefore lead to the depletion of $NADP^+$ in the chloroplast.

ROS are not only toxic molecules that must be removed to prevent the oxidative destruction of the cell, but are also key molecules in signal transduction that control many different cellular functions including various metabolic, physiological, and hormonal processes; developmental programs; defense responses against biotic and abiotic stresses; and systemic responses to environmental conditions (Mittler et al., 2004; Song et al., 2013; Noctor et al., 2014; Wendehenne et al., 2014; Mittler and Blumwald, 2015; Schmidt and Schippers, 2015; Inupakutika et al., 2016). Recently, it was proposed that a basal level of ROS is required to support the normal growth and development of the cell (Mittler, 2017). In addition, ROS are also actively and specifically formed in response to particular stimuli by specific ROS-producing enzymes such as NADPH oxidases, which are called respiratory burst oxidase homolog, and each is involved in a specific signal transduction process (Mittler et al., 2011; Vaahtera et al., 2014; Mignolet-Spruyt et al., 2016). It is now accepted that there are production site- and type-specific pathways for ROS signaling that allow plants to respond rigorously and flexibly to various environmental conditions (Gadjev et al., 2006; Vaahtera et al., 2014).

To avoid the cytotoxic effects of ROS while concomitantly maintaining the optimum levels of ROS required for them to exert their signaling roles, plants have developed rigorous antioxidant systems that are essential for their survival under changeable environmental conditions. The water-water cycle is as a unique plant-specific system that involves the production and scavenging of ROS in the chloroplasts (Asada, 1999; Shigeoka et al., 2002; Foyer and Shigeoka, 2011). This cycle, which is centered on the enzyme ascorbate peroxidase (APX), is also called the ascorbate-glutathione cycle, and plays a significant role in maintaining optimum H_2O_2 and superoxide levels (Asada, 1999). Since electrons are consumed in many steps of the process, including the photoreduction of oxygen and recycling of reductants, the water-water cycle acts not only as an antioxidant system but also as a system for the dissipation of excess electrons from PET in order to avoid the production of ROS, *i.e.*, as an electron sink. In addition to APX, thiol-peroxidases, peroxiredoxins (also known as thioredoxin peroxidase) and glutathione peroxidases are also involved in ROS metabolism in the chloroplasts (Dietz, 2011; Awad et al., 2015; Maruta et al., 2016a).

There are two important redox cofactors in the chloroplast that are known to be indispensable for it to function normally. The redox states and levels of NADP(H) are crucial factors in ROS homeostasis in the chloroplasts, because of their roles as electron acceptors in PET and as reducing equivalents for antioxidant systems (ascorbate- and thioredoxin-dependent pathways). In addition, flavins (flavin adenine dinucleotide, FAD; flavin mononucleotide, FMN; and their precursor riboflavin, RF) are essential redox cofactors that participate in many metabolic processes including photosynthesis and photoreception in plants. FAD in particular plays a role as the cofactor for enzymes involved in ascorbate biosynthesis and recycling and is, therefore, crucial for cell defense against oxidative stresses and programmed cell death (Mittler et al., 2004; Foyer and Noctor, 2005; de Pinto et al., 2006; Ishikawa and Shigeoka, 2008). For example, L-galactono-1,4-lactone dehydrogenase (which catalyzes the last step of the D-mannose/L-galactose pathway, the predominant ascorbate biosynthesis pathway in higher plants) is a flavoprotein associated with the mitochondrial respiratory chain (Smirnoff et al., 2001; Hancock and Viola, 2005; Leferink et al., 2008; Yoshimura and Ishikawa, 2017; Ishikawa et al., 2018). It has also been demonstrated an important role for flavin nucleotides at appropriate levels in the protection of plants from photo-oxidative damage (Ouyang et al., 2010). In addition, flavin nucleotides have been found to be associated with a variety of non-redox processes, such as light sensing and the photorepair of DNA (Sancar, 1994; Briggs and Huala, 1999; Losi and Gärtner, 2012). Therefore, the metabolism of these redox cofactors must be tightly regulated in cells. Generally, the balance between synthesis and degradation can significantly affect the intracellular levels of biomolecules. Although the biosynthetic pathways of NAD(P)H and FAD have already been well characterized (Noctor et al., 2006; Roje, 2007), the degradation processes and their physiological significance remained unclear until recently. However, studies on Nudix (Nucleoside diphosphate linked to some other moiety X) hydrolases (NUDXs), which exhibit pyrophosphohydrolase activity toward various nucleoside diphosphate derivatives, such as NAD(P)H and FAD, as well as an array of other nucleotides, have provided novel insights into the degradation process.

NUDXs are a diverse superfamily of proteins characterized by a conserved amino acid sequence $GX_5EX_7REUXEEXGU$ (Nudix motif), in which U is a hydrophobic residue (McLennan, 2006). They are distributed across all classes of organisms, including archaea, bacteria, yeast, algae, animals, and plants. Thousands of open reading frames encoding NUDXs in over 360 different species, have been identified by bioinformatics analysis (Gunawardana et al., 2009; Yoshimura and Shigeoka, 2015; Ishikawa et al., 2016). These enzymes have the potential to hydrolyze a wide range of organic pyrophosphates, including nucleoside di- and triphosphates, nucleotide cofactors, nucleotide sugars, and RNA caps. Some non-nucleotide molecules have also been identified as substrates for NUDXs, and other relevant substrates may also exist (Yoshimura and Shigeoka, 2015). Since these are signaling molecules, metabolic intermediates, and/or enzymes cofactors, as well as potentially toxic compounds, NUDXs are predicted to be important in controlling the levels of these compounds in the cells. In plants, *Arabidopsis thaliana*, *Oryza sativa*, *Populus trichocarpa*, *Vitis vinifera*, *Solanum lycopersicum,* and *Hordeum vulgare* possess 28, 30, 53, 30, 32, and 14 *NUDX* genes, respectively (Kraszewska, 2008; Tanaka et al., 2015; Yoshimura and Shigeoka, 2015; Ishikawa et al., 2016). NUDXs in *Arabidopsis* (AtNUDXs) can be classified into three types according to their predicted subcellular localization: the cytosol (AtNUDX1 to 11 and 25, and AtDCP2), mitochondrion (AtNUDX12 to 18), or chloroplast (AtNUDX19 to 24, 26, and 27). The *in vitro* activities of all AtNUDXs toward various substrates have been tested using recombinant proteins (Ogawa et al., 2005; Muñoz et al., 2006; Xu et al., 2006; Ogawa et al., 2008). The enzymatic properties of AtNUDXs with activities toward 8-oxo-(d)GTP (AtNUDX1), ADP-ribose (AtNUDX2), NAD(P)H (AtNUDX6, AtNUDX7, and AtNUDX19), GDP-mannose (AtNUDX9), CoA and its derivatives (AtNUDX11 and 15), ADP-glucose (AtNUDX14), Ap_nA (AtNUDX13 and AtNUDX25-27), FAD (AtNUDX23), and capped mRNA (AtDCP2) have been characterized. Recently, Karačić et al. (2017) reported that AtNUDX3, which contains the dipeptidyl peptidase III (DPP III) domain at the C-terminus, has DPP III activity against dipeptidyl-2-arylamide substrates, as well as pyrophosphohydrolase activity against isopentenyl diphosphate (IPP), a universal precursor for the biosynthesis of isoprenoid compounds. In addition, there is increasing evidence from functional analyses of these AtNUDXs that the physiological functions of NUDXs extend into many aspects of the regulation of cellular responses, including intracellular redox statuses, thereby helping to clarify the functions of their respective orthologs in other organisms. Detailed information on these AtNUDXs has been summarized in a recent review paper (Yoshimura and Shigeoka, 2015; Ishikawa et al., 2016).

This review summarizes the physiological roles of NUDXs, mainly chloroplastic AtNUDXs, as modulators of cellular redox states through their NAD(P)H and FAD pyrophosphohydrolase activities, and introduces recent progress in the study of NUDXs in various organisms.

2. Roles of NAD(P)H pyrophosphohydrolases in biotic and abiotic stress responses

Among the 28 AtNUDXs observed so far, at least three AtNUDXs (AtNUDX6, 7, and 19) have been demonstrated to exhibit

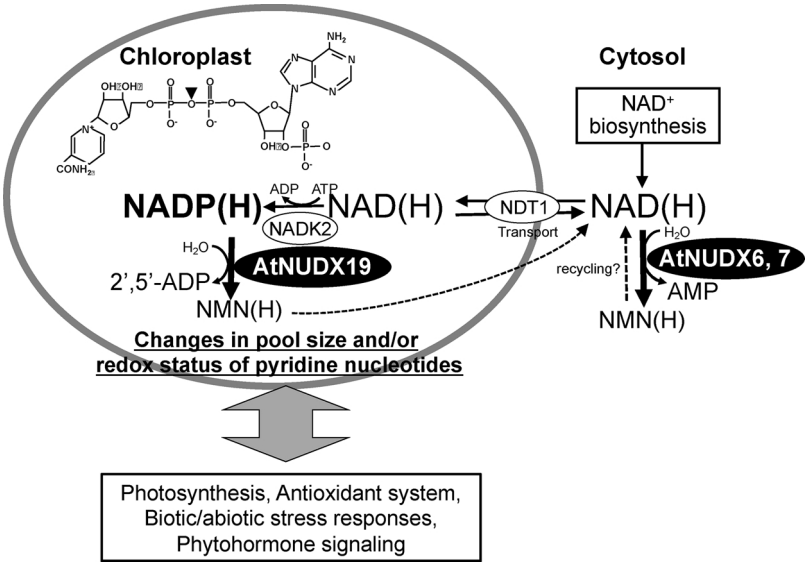

Fig. 1. Metabolic pathway for pyridine nucleotides in plants.

pyrophosphohydrolase activity toward NAD(P)H *in vivo*, suggesting the importance of the degradation of NAD(P)H in plant cells (Fig. 1). The pyridine nucleotides, such as NAD(P)H, are ubiquitous co-factors that are required for hundreds of redox reactions, and in synthetic pathways such as the dark reaction sugar syntheses in plants. Indeed, the excess accumulation of NADP(H) by the overexpression of the *Arabidopsis NADK2* gene, encoding the chloroplastic NAD kinase required for *de novo* $NADP^+$ synthesis from NAD^+, have been reported to cause accumulation of several metabolites associated with the Calvin cycle, accompanied by an increase in overall Rubisco activity in *Arabidopsis* and rice (Takahashi et al., 2009; Takahara et al., 2010). The pyridine nucleotides are also required for the regulation of various cellular processes, including transcription and microtubule metabolism, through NAD-deacetylation, mono or poly(ADP-ribosyl)ation, and intracellular Ca^{2+} signaling *via* NAD-derived cyclic ADP-ribose (Hunt et al., 2004; Noctor et al., 2006; Hashida et al., 2009; Briggs and Bent, 2011). Furthermore, NADP(H) is involved in the regulation of both the production and scavenging of ROS in plant chloroplasts. Decreases in the levels of $NADP^+$ and the redox states of NADPH/$NADP^+$ in the chloroplasts cause the overproduction of ROS in the PET system (Asada, 1999; Maruta et al., 2016a). In addition, NADPH serves as a reductant for antioxidant systems (Foyer and Shigeoka, 2011). Therefore, pyridine nucleotides are important not only for normal cellular processes but also cellular responses to various types of biotic and abiotic stresses which directly and indirectly impact on the cellular redox states. This implies that the regulation of intracellular pyridine nucleotide levels is critical for the accurate accomplishment of various cellular processes in plants.

Subcellular levels of both NADH and NAD^+ in *Arabidopsis* have been reported to increase or decrease under biotic and abiotic stresses, respectively (Ishikawa et al., 2009; Ogawa et al., 2009; Pétriacq et al., 2012). These findings suggest that increases in NAD(H) trigger the activation of various defense responses to protect the organism from biotic and abiotic stresses. In fact, transient increase in NAD^+ and NADH levels using an inducible NAD^+ overproduction system in *Arabidopsis* using *nadC* gene caused the accumulation of conjugated and free salicylic acid (SA) pools, resulting in the enhancement of resistance to infection by avirulent pathogens (Noctor et al., 2011; Pétriacq et al., 2012). The expression of AtNUDX7 with NADH pyrophosphohydrolase activity was induced by various types of biotic (avirulent, virulent, and non-host pathogenic attacks) and abiotic stresses (drought, salinity, wounding, and high light conditions), and treatment with various oxidants (paraquat, ozone, O_2^-, and H_2O_2) (Ishikawa et al., 2009; Yoshimura and Shigeoka, 2015). In contrast, the expression of AtNUDX6, its closest homologue, was only induced by avirulent pathogen attack, and by treatments with SA and H_2O_2 (Ishikawa et al., 2010; Yoshimura and Shigeoka, 2015; Ishikawa et al., 2016). These both suggest what the contribution of AtNUDX6 and 7 to intracellular NADH metabolisms may be. In fact, the use of plants in which the genes that encode AtNUDX6 and 7 were constitutively overexpressed and/or deleted has demonstrated that AtNUDX6 and 7 contribute to the modulation of various defense responses, such as the poly(ADP-ribosyl)ation (PAR) reaction and SA-induced Nonexpresser of Pathogenesis-Related genes 1 (NPR1)-dependent defense pathway against biotic and abiotic stresses (Yoshimura and Shigeoka, 2015; Ishikawa et al., 2016). That is, overexpression and knockout (KO) plants of the AtNUDX7 gene showed increased and decreased tolerance, respectively, to oxidative stress caused by paraquat treatment (Ishikawa et al., 2009). In the AtNUDX7-overexpressing plants, but not in the AtNUDX6-overexpressing plants, the PAR reaction was hyperactivated under oxidative stress, while the depletion of NAD^+ and ATP due to the activation of the PAR reaction was completely suppressed. These findings indicate that AtNUDX7 is involved in the regulation of defense mechanisms against oxidative DNA damage caused by paraquat treatment through modulating the PAR reaction (Ishikawa et al., 2009). On the other hand, the KO-*nudx7* plants exhibited enhanced resistance to both virulent and avirulent pathogenic strains (Bartsch et al., 2006; Ge et al., 2007; Straus et al., 2010). That is, AtNUDX7 also functions as a negative regulator of biotic stress responses by suppressing the excess accumulation of SA. However, the direct actions of AtNUDX7 on the PAR reaction and the SA accumulation have not yet been established.

It has been demonstrated that the SA-induced NPR1 activation, which is achieved by an oligomer-to-monomer reaction, is regulated by thioredoxins (TRXs) including TRX-h5 through the reduction or oxidation of its intermolecular disulfide bonds (Tada et al., 2008). The expression of *TRX-h5* and SA-induced NPR1-dependent genes was significantly suppressed and enhanced in SA-treated KO-*nudx6* and AtNUDX6-overexpressing plants, respectively (Ishikawa et al., 2010). This indicates that AtNUDX6 is involved in the biotic stress response as a positive regulator of NPR-1-dependent SA signaling pathway. Fonseca and Dong (2014) reported that AtNUDX8 is also involved in SA signaling and biotic stress response as a positive regulator. The expression of *TRX-h2* and *TRX-h3* as well as *TRX-h5* was suppressed in KO-*nudx8* plants grown under normal and SA-treated conditions. However, the enzymatic activity of AtNUDX8 toward any nucleoside diphosphate derivatives as substrates is undetectable, although the protein has

moderate similarity to AtNUDX6 and 7.

Interestingly, some pathogens may have developed a strategy to suppress plant immunity by secreting a NUDX enzyme exhibiting ADP-ribose/NADH pyrophosphohydrolase activity (Dong et al., 2011). Recently, a plant prolyl-peptidyl isomerase (PPIase), cyclophilin, has been reported to activate the enzymatic activity of Avr3b, a *Phytophthora sojae* (an oomycete pathogen) RXLR effector having NADH pyrophosphohydrolase activity, after it is delivered into plant cells; that is, cyclophilin functions as a helper required for the avirulence and virulence functions of Avr3b (Kong et al., 2015).

Recently, mouse Nudt13 protein, having NAD(P)H pyrophosphohydrolase activity, has been reported to localize mitochondria, suggesting that Nudt13 acts to regulate the concentration of mitochondrial reduced pyridine nucleotide cofactors and the $NAD(P)^+$/NAD(P)H ratio (AbdelRaheim et al., 2017). Shimizu et al. (2012) have demonstrated that the NADH pyrophosphohydrolase in *Aspergillus nidulans*, NdxA, controls intracellular NAD(H) levels and negatively regulates sirtuin function and chromatin structure. It has also been found that NdxA controls glycolysis by controlling cellular NADH and/or ADP-ribose levels under O_2-limited conditions (Shimizu and Takaya, 2013; Shimizu, 2018).

A transient expression analysis of the active and inactive forms of AtNUDX6 and 7 has clearly demonstrated the importance of AtNUDX6 and 7 in the physiological roles of NADH metabolisms (Ogawa et al., 2016). The transient expression of active and inactive AtNUDX6 proteins induced the expression of *TRX-h5*, the activator of NPR1 and SA-induced NPR1-dependent defense genes, while the active and inactive AtNUDX7 proteins suppressed the accumulation of SA and subsequent gene expression. This indicates that AtNUDX6 and 7 proteins themselves play distinct roles in stress responses, irrespective of the NADH metabolism. The protein RACK1 A (Receptor for Activated C Kinase 1 A) and AGG1 and AGG2 gamma subunits of the signal-transducing heterotrimeric G protein have been identified as AtNUDX7 interactors using a yeast two-hybrid system, suggesting the formation of regulatory proteins is involved in signal transduction (Olejnik et al., 2011). These findings indicate that particular interactors are required for the intracellular function of AtNUDX6 and 7. In contrast, the transient expression of active AtNUDX6 and 7 proteins suppressed NADH levels and induced PAR activity, whereas that of their inactive forms did not. This indicates that the regulation of NADH metabolism is involved in the modulation of the PAR reaction. The genes (NADH-responsive genes, NRGs) whose expression levels correlated with intracellular NADH levels have been identified by a transcriptome analysis using the wild-type, KO-*nudx6*, KO-*nudx7*, and double KO-*nudx6/7* plants, in which intracellular NADH levels increased stepwisely (Ogawa et al., 2016). More than half of $NRGs^+$, whose expression were positively correlated with NADH levels, did not overlap with the genes responsive to chloroplast-derived H_2O_2, $^1O_2$, $O_3{}^+$, or ascorbate (op den Camp et al., 2003; Kerchev et al., 2011; Short et al., 2012; Sewelam et al., 2014). Pétriacq et al. (2016) reported that NAD^+ triggers the production of ROS and defense hormones and reorchestrates the defense metabolome. These findings indicate a novel role for intracellular pyridine nucleotide levels as an integral regulator of broad-spectrum defense systems in plant cells.

Chloroplastic AtNUDX19 has been reported to effectively hydrolyze NADPH rather than NADH (Fig. 1; Ogawa et al., 2008). Under normal and high light conditions, intracellular NADPH levels (but not NADH) of knockdown (KD)- and KO-*nudx19* plants were significantly higher than those of the wild-type plants (Maruta et al., 2016b). In contrast, levels of $NADP^+$ were lower in the KD- and KO-*nudx19* plants than in the wild-type plants, resulting in a decrease in total NADP(H) under normal light conditions. Corpas et al. (2016) have demonstrated that the activities of the enzymes involved in the production of NADPH from $NADP^+$, such as NADP-isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and NADP-malic enzyme in the leaves and roots of KO-*nudx19* plants were higher than those of the wild-type plants under normal conditions. Therefore, it is likely that these enzymes are activated to produce more NADPH in the KO-*nudx19* plants, resulting in decreases in $NADP^+$. This implies that AtNUDX19 has significant impacts on the cellular levels and redox states of pyridine nucleotides in plant cells. The ratio of NADPH/NADP(H) in KD- and KO-*nudx19* plants was approximately 2-fold higher than that in the wild-type plants under normal and high light conditions (Maruta et al., 2016b). In general, the high reduction states of NADPH can excessively reduce PET, thereby enhancing ROS production (Asada, 1999). Therefore, KD- and KO-*nudx19* plants thought to be sensitive to photooxidative stresses. However, the growth of KD- and KO-*nudx19* plants under moderate light conditions was unexpectedly, but clearly, greater than that of the wild-type plants (Maruta et al., 2016b). In addition, the KD- and KO-*nudx19* plants showed higher tolerance than the wild-type plants to the oxidative stress caused by paraquat treatment, suggesting these plants to be insensitive to oxidative stresses. The increase in 1-*q*P (the reduction states of PSII) and the decrease in øPSII (the quantum yield of photosystem II) under high light irradiation were mitigated in the KO- and KD-*nudx19* plants, indicating that the KD- and KO-*nudx19* plants could prevent the excessive reduction of PET more than wild-type plants could (Maruta et al., 2016b). The initial activities of sedoheptulose-1,7-bisphosphatase and fructose-1,6-bisphosphatase, which are involved in the Calvin cycle and are tightly controlled through thiol-dependent redox regulation, were significantly higher in the KD- and KO-*nudx19* plants than in the wild-type plants during high light exposure (Maruta et al., 2016b). In line with this, the carbon assimilation rate was also higher in the KD- and KO-*nudx19* plants than in wild-type plants above a light intensity of 200 µmol photons m^{-2} s^{-1}, especially at a saturating irradiance (1200 µmol photons m^{-2} s^{-1}). Although how these were activated in the KD- and KO-*nudx19* plants is currently unknown, the activation of photosynthesis is also observed in transgenic *Arabidopsis* and rice lines overexpressing chloroplastic *NADK2* (Takahashi et al., 2009; Takahara et al., 2010). Assuming that photosynthesis is enhanced through the same mechanism in both *nudx19* plants and *NADK2*-overexpressors, these findings suggest that accumulation of NADP(H) is critical for the enhancement of photosynthesis. In addition, the total activities of antioxidative enzymes, APX, dehydroascorbate reductase, and monodehydroascorbate reductase were slightly but significantly higher under high light conditions in the KO- and KD-*nudx19* plants than in the wild-type plants, suggesting that accumulation of NADP(H) activates antioxidative capacities under high light. These findings indicate that AtNUDX19 acts as a negative regulator of oxidative stress tolerance by modulating the NADP(H) pool size and/or redox status in plant chloroplasts (Fig. 1).

The chloroplastic AtNUDX19 and cytosolic AtNUDX6/7 hydrolyze NADPH to NMNH and 2′,5′-ADP and NADH to NMNH and AMP, respectively. Dotted arrows indicate the putative NMN(H) recycling steps. Arrowhead in the chemical structure of NADPH indicates the site of hydrolysis reaction by AtNUDX19. Changes in pool size and/or redox status of pyridine nucleotides drive various cellular processes and stress responses and they mutually affect each other. NDT1; NAD^+ carrier (Palmieri et al., 2009); NMNH; reduced nicotinamide mononucleotide.

The AtNUDX19 protein (438 a.a.) possesses a chloroplast-targeting signal at N-terminus (36 a.a.), and its full length molecular mass is 48.3 kDa (without the targeting signal; 44.5 kDa) (Ogawa et al., 2008). According to the Pfam database, AtNUDX19 consists of three domains; *i.e.*, NADH pyrophosphatase-like rudimentary NUDIX domain (NUDIX-like, PF09296, residues 87–205), NADH pyrophosphatase zinc ribbon domain (zf-NADH-PPase: PF09297, residues 209–240), and NUDIX domain (PF00293, residues 243–369)(Maruta et al., 2016b). In addition, AtNUDX19 has the SQPWPFPxS motif immediately downstream of the Nudix motif within the NUDIX domain. This motif is found in the NADH pyrophosphohydrolases such as human NUDT12, *E. coli* Orf257, and yeast Npy1 proteins, suggesting its role on the activity (Frick and Bessman, 1995; AbdelRaheim et al., 2001, 2003). AtNUDX19 homologs are found to widely distribute in photosynthetic eukaryotes, including

algae, and moss as well as various higher plant species (Maruta et al., 2016b). This implies the importance of NADP(H) hydrolysis in the chloroplasts.

It has also been reported that the overexpression of the *Arabidopsis NADK2* enhances tolerance to oxidative damage in rice (Takahara et al., 2010), whereas *Arabidopsis* KO-*nadk2* mutants show increased sensitivity to stress treatments (Chai et al., 2005; Takahashi et al., 2006). Recently, KO-*nadk2* mutants were reported to show high sensitivity to drought stress caused by impairments of abscisic acid (ABA)-induced stomatal closure; ABA inhibition of light-promoted stomatal opening; and ABA-stimulated accumulation of H_2O_2, Ca^{2+}, and nitric oxide (NO) in guard cells (Sun et al., 2017). More recently, it has been reported that NADK2 is regulated by light and redox status, and that the ferredoxin/thioredoxin system, a well-known redox-active protein cascade that transfers reducing equivalents to various enzymes, regulates the chloroplastic $NADP^+$ status and NADPH pool size (Hashida et al., 2018). Thus, it is clear that NADPH levels and redox status in the chloroplasts have a significant impact on the various cellular processes, such as photosynthesis, antioxidant systems, and stress responses (Fig. 1).

A microarray analysis using the wild-type and KO-*nudx19* plants has revealed further roles for NADPH metabolism in chloroplasts (Maruta et al., 2016b). The expression of the SA-responsive genes *PR1* and *PR2* were up-regulated in the KO-*nudx19* plants. The expression levels of *SYSTEMIC ACQUIRED RESISTANCE DEDICIENT1* (*SARD1*) and the *WRKY38* transcription factors, which are known to be involved in the SA-dependent pathogen response, also increased in the KO-*nudx19* plants. The KO-*nudx19* plants accumulated significant amounts of free SA and were more sensitive to treatment with SA than the wild-type plants. The expression of *AtNUDX19* was also responsive to the treatment with SA, indicating that AtNUDX19 acts as a negative regulator of SA synthesis. In contrast, the KO-*nudx19* plants were mildly insensitive to jasmonic acid (JA), and ABA, suggesting that AtNUDX19 is involved in JA- and ABA-mediated pathways possibly through the regulation of SA synthesis. Since both chloroplastic AtNUDX19 and cytosolic AtNUDX7 were found to regulate SA-dependent and SA-independent defense signaling pathways (Bartsch et al., 2006; Ge et al., 2007), it is suggested that the pyridine nucleotides-mediated regulation of plant defense involves SA-dependent and SA-independent signaling mechanisms. Indeed, an increase in intracellular NAD^+ levels in transgenic *nadC* plants, which are plants that overproduce inducible NAD^+, enhanced resistance against a diverse range of virulent pathogens (Pétriacq et al., 2016). In addition, elevated NAD^+ levels induced the accumulation of defense regulatory phytohormones such as SA, JA, and ABA, as well as the production of ROS derived from mitochondrial respiration and the expression of redox marker genes in the cytosol and mitochondria.

Taken together, it is clear that modulation of the levels of NADP(H) and its redox status in the chloroplasts are indispensable in the fine-tuning of stress responses through the regulation of photosynthesis, the antioxidant system, and hormonal signaling (Fig. 1). In addition, the regulation of pyridine nucleotide metabolism in other cellular compartments, as well as in the chloroplasts, also seems to be important. However, the mechanisms involved are extremely complicated, since the changes in the pool size and redox status of pyridine nucleotides have a large impact on various cellular processes. As described above, in addition to the chloroplastic AtNUDX19, the other AtNUDXs that have NADH pyrophosphohydrolase activity, such as cytosolic AtNUDX6 and 7, are also involved in the photooxidative responses. Therefore, it would also be interesting to investigate the crosstalk between these AtNUDX isoforms with regards to the fine-tuning of stress and hormonal responses in future studies.

3. Roles of FAD pyrophosphohydrolases in flavin metabolism

The riboflavin (RF) derivatives FAD and FMN are critical redox cofactors in all living organisms. FAD and FMN are required for a wide variety of metabolic processes including mitochondrial electron transport, antioxidant reduction, protein folding, chromatin remodeling, as well as the metabolism of nucleotides, amino acids, other cofactors, and numerous other biologically important compounds (Lynch et al., 2018). Plants also require these cofactors for specialized functions such as blue-light signaling and photosynthesis. RF is synthesized *de novo* in plants, bacteria, and yeast; FMN is then synthesized from RF *via* ATP-dependent phosphorylation by RF kinases. FAD is subsequently formed from FMN *via* ATP-dependent adenylylation by FAD synthetases (Roje, 2007; Maruta et al., 2012; Lynch et al., 2018). Bioinformatic and proteome analyses have revealed that plant riboflavin biosynthesis occurs in the chloroplasts (Roje, 2007; Gerdes et al., 2012). However, information regarding the biosynthesis of FMN and FAD in plants is still limited. The activities of the respective enzymes have been detected in various plant species, some of which have been cloned and characterized from *Arabidopsis*. Interestingly, early studies have suggested that the enzymes that hydrolyze FAD to FMN and AMP exist in plants (Roje, 2007). In addition, Sandoval and Roje (2005) have characterized a cytosolic bifunctional enzyme from *Arabidopsis*. This enzyme has reversible activities: the riboflavin kinase activity and the FMN hydrolase activity. These results suggested that the levels of FMN and FAD are maintained by a balance between synthesis and hydrolysis in the cells.

Under such circumstances, it has been demonstrated that chloroplastic AtNUDX23 is the only AtNUDX enzyme that exhibits pyrophosphohydrolase activity towards FAD to hydrolyze FMN and AMP (Fig. 2; Ogawa et al., 2008). The expression levels of *AtNUDX23*, as well as that of the genes involved in flavin metabolism, significantly increased under continuous light conditions (Maruta et al., 2012). This lead to the accumulation of RF and FAD. Interestingly, the effects of the overexpression of the *AtNUDX23* gene on the expression of flavin metabolic genes were similar to that of its suppression; that is, intracellular levels of RF, FMN, and FAD decreased equally in both overexpressed and suppressed *Arabidopsis* plants. The treatment of *Arabidopsis* leaves with exogenous flavins caused changes in the expression levels of flavin metabolic genes, resulting in constant levels of FMN and FAD, but an overaccumulation of RF. Since the overaccumulation of multifunctional cofactors such as FMN and FAD is deleterious for cells, it is likely that excess amounts of such compounds must be expeditiously degraded into their precursor, RF. Although further studies are required to elucidate the underlying mechanism responsible for the regulation of flavin metabolism, these findings suggest that negative feedback regulation of flavin metabolism is essential to maintain the homeostasis of the essential cofactors, FMN and FAD. In the metabolic system, AtNUDX23 contributes by regulating the balance between FAD and FMN in chloroplasts through the hydrolysis of FAD (Fig. 2; Maruta et al., 2012). Homologs of AtNUDX23 are found to widely distribute in photosynthetic eukaryotes, but not in photosynthetic prokaryotes (Ogawa et al., unpublished data), implying the importance of FAD hydrolysis in the chloroplasts.

AtNUDX23 hydrolyzes FAD to FMN and AMP. Dotted arrows indicate the predicted membrane transport steps of flavins. Accumulation of flavins causes feedback inhibition of flavin metabolism. Question mark indicates unidentified enzyme or transporter. Arrowhead in the chemical structure of FAD indicates the site of hydrolysis reaction by AtNUDX23. Maintenance of flavin homeostasis is involved in various cellular processes and stress responses and they mutually affect each other. cpFHy1, chloroplastic FMN hydrolase (Rawat et al., 2011); RibF1, 2, chloroplastic FAD synthetases (Sandoval et al., 2008).

Recently, Lynch et al. (2018) identified a non-Nudix enzyme (Fpy1p) with FAD pyrophosphohydrolase activity from *Saccharomyces cerevisiae*. The recombinant Fpy1p exhibited the ability to hydrolyze FAD and other substrates, such as ADP-ribose and NADH at about half rate of FAD. Its enzymatic property was dependent on K^+ and divalent cations, with similar kinetic parameters to those of the other NUDXs that exhibit FAD pyrophosphohydrolase activity, including AtNUDX23.

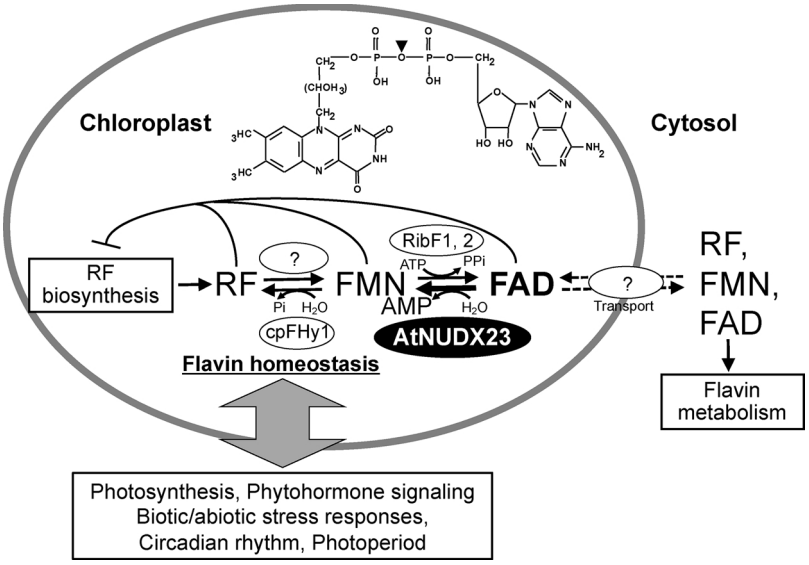

Fig. 2. Metabolic pathway for flavin nucleotides in plants.

AtNUDX23 had high affinity for FAD (K_m value, 9.1 ± 0.9 μM) in the presence of 5 mM Mg^{2+} (Ogawa et al., 2008). The K_m value of Fpy1p for FAD was calculated as 16.1 ± 2.1 μM and 19.0 ± 2.7 μM in the presence of 4 mM Co^{2+} and 8 mM Mg^{2+}, respectively (Lynch et al., 2018). These values were markedly lower than those of *Paenibacillus thiaminolyticus* YZGD (3700 μM) and T4 bacteriophage nudE.1 (1050 μM) (Tirrell et al., 2006; Xu et al., 2002). The k_{cat} values of AtNUDX23 and Fpy1p for FAD in the presence of Mg^{2+} were 0.08 s^{-1} and 0.20 s^{-1}, respectively. Therefore, the catalytic efficiency (k_{cat}/K_m) of Fpy1p (1.1 × 10^4 s^{-1} M^{-1}) was almost the same as that of AtNUDX23 (8.4 × 10^3 s^{-1} M^{-1}). Biochemical and functional studies using mutant yeast strains have suggested that Fpy1p has a vital role as a novel non-Nudix FAD pyrophosphohydrolase in the regulation of FAD and NAD(H) contents, in response to the metabolic needs of the cell during the stationary phase in yeast cells (Lynch et al., 2018). Homologs of Fpy1p exist in representative mammal, fish, invertebrate, algal, and dicot/monocot plant species including *Arabidopsis*. Since the Fpy1p homologue in *Arabidopsis* is predicted to localize in cytosol, but not in chloroplast (Ogawa et al., unpublished data), such non-Nudix pyrophosphohydrolase(s) may also be involved in FAD metabolism in cytosol of plant cells.

There is increasing evidence for the involvement of flavins in various cellular processes and responses in plants. An *Arabidopsis* photosensitive mutant (*phs1*) has been isolated and characterized (Ouyang et al., 2010). The *PHS1* gene encodes a putative protein with a RF deaminase-reductase domain, and the FAD contents of this mutant were notably lower than those of the wild-type plants. When the *phs1* mutant was grown under high light conditions, the mutant exhibited stunted growth and bleached leaves and a decrease in ferredoxin-$NADP^+$ oxidoreductase (FNR) activity, which requires FAD as a cofactor in the chloroplasts. The *phs1* mutant also suffered from severe photo-oxidative damage, with an increase in antioxidant enzyme activity and a drastic reduction of chlorophyll and photosynthetic protein levels observed. Exogenous FAD treatment rescued the photosensitive phenotype of *phs1* mutant, suggesting that the maintenance of constant levels of flavins is important for the protection of plants from photo-oxidative stresses. Furthermore, down-regulation of free flavins by the expression of turtle riboflavin-binding protein (RfBP) was found to be responsible for NADPH oxidase-independent H_2O_2 accumulation and the pathogen defense (Deng et al., 2011). *Arabidopsis* mutants that have a partial defect in *COS1*, which encodes the lumazine synthase involved in the RF biosynthesis, causing a partial decrease in RF content were compromised the regulatory role of JA in leaf senescence (Xiao et al., 2004). Moreover, externally applied RF induces resistance to pathogens by priming defense responses in a manner similar to that of SA dependence or independence, according to the type of pathogen (biotrophic or necrotrophic) encountered by the plant (Dong and Beer, 2000; Zhang et al., 2009). These results suggest that changes in the flavin levels, especially of RF, cause physiological and pathological responses by affecting redox and/or phytohormone signaling pathways (Fig. 2).

At present information on the regulatory factors involved in flavin homeostasis, as well as transporters for inter- and intracellular flavin transport, is limited. Recently, we identified novel factors involved in flavin metabolism using a transcriptome analysis on leaves that had been treated with exogenous FAD (Ogawa et al., unpublished data). This study revealed that the expression of 262 and 198 genes were either up-regulated and down-regulated, respectively, by the exogenous FAD treatment. Of these, there were 47 and 17 genes that encoded for putative transcription factors and transporters, respectively. Interestingly, many genes that encode for clock-related transcription factors such as *CIRCADIAN CLOCK-ASSOCIATED1* (*CCA1*) and *PSEUDO-RESPONSE REGULATOR* (*PRR*) family were included. Ji et al. (2014) demonstrated that a decrease of free flavin levels in leaves caused by the expression of RfBP in transgenic *Arabidopsis* induces an early flowering phenotype. It also affects the expression of photoperiod and flowering time genes, including *CCA1*. These findings suggest that intracellular levels of flavins participate in the regulation of the circadian rhythm and photoperiod in plant cells (Fig. 2). Further analyses will uncover the regulatory mechanisms that control the homeostasis of flavins as a multifaceted player in plants.

4. Recent progress in the studies of NUDXs in various organisms

Over the past 20 years, the molecular and enzymatic properties of NUDXs in various organisms, mainly in mammalian and bacteria, have been uncovered and summarized by previous reviews (Bessman et al., 1996; Taddei et al., 1997; McLennan, 2006, 2013). Among them, *E. coli* MutT and its orthologs in other organisms have been characterized as antimutators. These MutT-type NUDXs hydrolyze both oxidized deoxyribonucleotides and ribonucleotides, such as 8-oxo-(d)GTP and 2-OH-(d)ATP, and play a major role in the prevention of mutations caused by the misincorporation of oxidized nucleotides into DNA and RNA. Mammalian cells contain multiple MutT-type NUDXs, such as MTH1-3 and NUDT5 (McLennan, 2006). Human MTH1 has both 8-oxo-(d)GTP and 2-OH-(d)ATP pyrophosphohydrolase activities and protects the cells from H_2O_2-induced cell dysfunction and death by hydrolyzing

oxidized purine nucleotides (Nakabeppu et al., 2010). In addition, many NUDXs with pyrophosphohydrolase activities toward the other nucleotide diphosphate derivatives have been identified and enzymatically characterized from various organisms. As described above, examples of these include the enzymes that hydrolyze NAD(P)H from *Escherichia coli*, *Saccharomyces cerevisiae*, human, mouse, and fungi, and *Arabidopsis* (Frick and Bessman, 1995; Xu et al., 2000; AbdelRaheim et al., 2003; Ogawa et al., 2005, 2008; AbdelRaheim et al., 2017; Shimizu et al., 2012) and FAD from *Paenibacillus thiaminolyticus*, T4 bacteriophage, and *Arabidopsis* (Tirrell et al., 2006; Xu et al., 2002; Ogawa et al., 2008). However, with only a few exceptions, the physiological roles of NUDXs with NAD(P)H and FAD pyrophosphohydrolase activities have largely remained unclear in various organisms except for plants.

Since either possible or demonstrated roles of NUDX having activity toward the other substrate in various organisms have been summarized by previous reviews (McLennan, 2006, 2013), novel information on the enzymatic properties and physiological roles of these NUDXs, which were uncovered by recent studies, were summarized here. Nguyen et al. (2016) reported the substrate specificity for 8 putative NUDX enzymes from *Bifidobacterium adolescentis*, *Bacteroides fragilis*, *Bacillus halodurans*, *Clostridium perfringens*, *Listeria innocua*, *Methanosarcina mazei*, and *Streptococcus suis*. O'Handley et al. (2016) demonstrated the kinetic and mutational analyses of the ADP-ribose pyrophosphohydrolase from *Mycobacterium tuberculosis*. In ascomycete yeast, *S. cerevisiae*, Ysa1p has been reported to catalyze the degradation of ADP-ribose or O-acetyl-ADP-ribose (OAADPr) and play a key role in modulating intracellular levels of these compounds (Tong et al., 2009). Since ADP-ribose and OAADPr counteract the increased levels of ROS through the inhibition of complex I of the electron transport chain in mitochondria and re-routing of glucose metabolism from the glycolytic pathway to the pentose phosphate pathway, the deletion of the *YSA1* gene resulted in enhanced cellular resistance to oxidative stress (Tong et al., 2009). On the other hand, a Ysa1p ortholog in basidiobycete yeast, *Cryptococcus neoformans*, *ysa1* mutants did not show enhanced tolerance to oxidative damage agents, such as H_2O_2 and menadione, but exhibited increased sensitivity to a thiol-specific oxidant (Lee et al., 2014), suggesting that the role of NUDX in each organism is evolving in a species specific manner. The NUDX enzyme (TsNd) from *Trichinella spiralis*, an ovoviviparous nematode parasite, has been reported to have pyrophosphohydrolase activity toward dGTP, NAD, NADP, and CoA (Long et al., 2015). TsNd was able to bind specifically to mouse intestinal epithelial cells (IECs) and promoted the larval invasion of IECs, whereas anti-TsNd antibodies inhibited the larval invasion of IECs. These results suggested that the TsNd protein is able to interact with host IECs and the enzymatic activity is essential for the *T. spiralis* larval invasion, development, and survival in host.

In mammals, the biological functions of many NUDXs remain elusive and several members are completely uncharacterized. Previous studies on individual NUDX proteins in model organisms have provided some insights, but the key physiological roles of these enzymes, with the exception of MTH1, have yet to be revealed. Mammalian NUDT16 protein with strong (deoxy)inosine di- and triphosphate (IDP and ITP) hydrolyzing activity has been reported to play a protective role in maintaining chromosome stability and normal cell growth by the elimination of (d)IDP and (d)ITP from nucleotide pools (Abolhassani et al., 2010). Some NUDXs have recently been reported to be up-regulated following cellular stresses, and may be important for survival of cells under these conditions. They are therefore potentially good targets for therapeutic intervention, such as killing cancer cells (Carreras-Puigvert et al., 2017). Moriyama et al. (2016) have systematically identified NUDT15 variants associated with thiopurine disposition and host toxicity, characterized their enzymatic properties, and comprehensively investigated the molecular pathways linking NUDT15 to thiopurine toxicity. These results indicate that a comprehensive pharmacogenetic model integrating NUDT15 variants may inform personalized thiopurine therapy (Moriyama et al., 2016). However, studying individual NUDXs is difficult due to the numerous substrates and functional redundancies. To address this, Carreras-Puigvert et al. (2017) used a family-wide approach by building the largest collected set of information to date on all human NUDXs, including biochemical, structural, genetic, and biological properties, and used a novel algorithm, FUSION, to investigate their similarities. They purified 18 human NUDXs and screened 52 substrates to provide a substrate redundancy map, and to link structure and activity relationships. The global expression analysis revealed that human NUDXs were significantly highly expressed in adrenal-, endometrium-, and lung-related cancers, whereas it was the opposite in kidney-and testis-related cancer. In addition, the KD of several NUDXs altered the cell cycle distribution and affected cell viability, but mainly in cancer cells. Importantly, all data were integrated by the FUSION algorithm to create a comprehensive human NUDX profile map. This comprehensive and exhaustive analysis of the human NUDXs provides a novel insight into their substrate selectivity and biological functions, and a plethora of data ranging from gene and protein expression and substrate specificity to functional genomics (Carreras-Puigvert et al., 2017).

As described above, AtNUDX7 plays a role in stress response, possibly through interactions with other regulatory proteins (Olejnik et al., 2011; Ogawa et al., 2016). Recently, we identified one of the small GTPase proteins as an AtNUDX6 interactor using yeast two-hybrid screening (Ogawa et al., unpublished data). As with AtNUDX6 and 7, the existence and importance of the interaction partner of NUDXs has been revealed in other organisms. Human NUDT2 was identified as a novel binding protein of Rag GTPases. It is also a positive regulator of the mammalian target of rapamycin complex 1 (mTORC1) signaling pathway, a well-known molecular sensor of amino acid levels (Kwon et al., 2017). NUDT2 interacted with Rag GTPases, and this interaction was required for the translocation of mTORC1 to the lysosomal membrane. NUDT2-silenced cells impaired the activation of mTORC1 and were arrested in the G0/G1 phases, indicating NUDT2-dependent mTORC1 regulation is critical for proliferation of breast cancer cells. Previously, it has been reported that NUDT2 asymmetrically hydrolyzes Ap_4A into ATP and AMP. This process may have important roles in critical physiological events such as apoptosis, DNA repair, transcription, and immune responses (Kisselev et al., 1998; McLennan, 2000; Carmi-Levy et al., 2008). Interestingly, however, the binding affinities of NUDT2 to Rag GTPases were independent of its hydrolase activity, and the expression of an enzymatically inactive form of NUDT2 did not alter the activation of mTORC1 signaling. This indicates that Ap_4A hydrolysis of NUDT2 is not required for recruitment of mTORC1 to the lysosomal membrane (Kwon et al., 2017). These findings strongly suggest that organisms have developed the functions of NUDXs not only as the hydrolysis enzyme regulating various metabolisms, but also as the regulator involved in signal transduction processes.

5. Conclusions and perspectives

Previous studies on the biological and physiological functions of NUDXs partially revealed their roles in cellular housecleaning and in the control of metabolic pathways. Recently, they have also been demonstrated to be involved in the regulation of diverse cellular responses including biotic/abiotic stress responses, through the hydrolysis of various nucleotide derivatives, including redox cofactors. In the plant chloroplasts especially, AtNUDX19 and 23 play key roles in the modulation of the pool size and redox status of NADP(H) and flavin nucleotides, respectively. Changes in the levels and redox status of cofactors NADP(H) and flavin nucleotides in the chloroplasts have been demonstrated to have multiple effects on photosynthesis, ROS metabolism, biotic/abiotic stress responses, phytohormone signaling pathway, and photoperiods. However, since these cofactors are multifunctional molecules, more research is required to better understand their role in the coordination of various physiological processes.

Intricate cooperative regulation of the levels and redox statuses of NADP(H) and flavin nucleotides may be required for growth and survival strategies in plant cells. Further comprehensive understanding of the biosynthesis, degradation, and transport of such redox cofactors will fully uncover the physiological importance of the modulation of the subcellular levels of these nucleotide cofactors and the role of NUDXs in chloroplasts.

CRediT authorship contribution statement

Takahisa Ogawa: Conceptualization, Data curation, Validation, Funding acquisition, Writing - original draft. **Kazuya Yoshimura:** Conceptualization, Project administration, Validation, Funding acquisition, Writing - review & editing.

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