

## MINI REVIEW

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## Apyrases in *Arabidopsis thaliana*

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### Abstract

Apyrases belong to the ATPase family of enzymes that hydrolyze phosphoanhydride bonds of nucleoside tri- and diphosphates. These enzymes differ markedly from other phosphohydrolases due to their high specific activity, broad divalent cation requirement, broad nucleotide substrate specificity, and insensitivity to various inhibitors. In the past 30 years, apyrases have been frequently studied in mammals. In comparison, research of apyrases in plants has received little attention, despite the growth of plants being closely related to the apyrases. In this review, we summarize the research of the apyrases in *Arabidopsis thaliana* and point to the possible future directions of research. Apyrases have seven members found in *Arabidopsis thaliana*, each with different properties and functions. Currently, the characterization and functions of AtAPY1 and AtAPY2 have been reported, though, to the best of our knowledge, the other apyrase members (AtAPY3 to 7) have not yet been sufficiently described. In this review, we also summarize the progress being made and the difficulties encountered in apyrase research in *Arabidopsis thaliana*.

*Additional key words:* ATPase family, AtAPY1 and AtAPY2, enzyme localizations and biochemical properties

### Introduction

Apyrases are calcium-activated enzymes that catalyze the conversion of adenosine triphosphate (ATP) to adenosine diphosphate (ADP), adenosine monophosphate (AMP), and Pi (Veloria *et al.* 2017), and belong to the guanosine diphosphatase 1 - CD39 nucleoside phosphatase superfamily. They contain five apyrase conserved regions (ACRs) (Chiu *et al.* 2015), which are the main characteristics of the apyrases. Apyrases have been found in all prokaryotes and eukaryotes (Komoszyński and Wojtczak 1996). They are widely distributed in both animal and plant tissues and can be classified as endo-apyrase or ecto-apyrase based on their localization and biochemical properties (Plesner 1995, Komoszyński and Wojtczak 1996). According to Knowles (2011), apyrases are multifunctional enzymes involved in pathogen-host

interactions, plant growth, lipid and protein glycosylation in cells, and oncogenesis (Clark *et al.* 2010). Their diverse expressions (Day *et al.* 2000) and membrane and subcellular localization (Cohn *et al.* 2001, Govindarajulu *et al.* 2009) also hint at their roles in various metabolic processes (Sunhee *et al.* 2009, Kavaiool and Ezhova 2010). In the past 30 years, apyrases have been studied in many eukaryotic systems and examined for their possible use (Moustafa 2014, Veloria *et al.* 2017).

In plants, despite the lack of understanding about the functions and regulation of the apyrases, some progress is being made in elucidation of the the roles of plant apyrases in phosphate transport (Thomas *et al.* 1999, Clark *et al.* 2010) cytoskeleton-based cellular metabolism (Shibata *et al.* 1999, Chen *et al.* 2013), toxin resistance

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Submitted 13 November 2017, last revision 20 June 2018, accepted 16 July 2018.

*Abbreviations:* AtAPY - *Arabidopsis thaliana* apyrase; ECM - extracellular matrix; GDP - guanosine diphosphate; NDP - nucleoside diphosphate; NMP - nucleoside monophosphate; NTP - nucleoside triphosphate; NTPDase - nucleoside triphosphate diphosphohydrolase.

*Acknowledgements:* This work was funded by the National Natural Science Foundation of China (No. 11572063) and the Graduate Research and Innovation Foundation of Chongqing (No. CYS18016).

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(Thomas *et al.* 2000), nodulation (Etzler *et al.* 1999, Govindarajulu *et al.* 2009), and growth (Wu *et al.* 2007, Riewe *et al.* 2008, Yuo *et al.* 2009). Plant apyrases hydrolyze the di- and tri-phosphate bonds of nucleotides in the presence of divalent metal ions according to equations:  $ATP + 2 H_2O \rightarrow AMP + 2P_i$  and  $ADP + H_2O \rightarrow AMP + P_i$  (Kettlun *et al.* 1982, Wujak and Komoszyński 2011, Wujak *et al.* 2013). Apyrases do not hydrolyze ester bonds and inorganic pyrophosphate (Handa and Guidotti 1996, Komoszyński 1996). In contrast to ATPases, low substrate specificity and insensitivity to classical inhibitors of ATPases are two main characteristics of the apyrases (Komoszyński and Wojtczak 1996).

Various researchers have highlighted two main categories of apyrases: 1) ecto-apyrases that are present in the extracellular matrix (Plesner 1995), and 2) endo-apyrases that are localized in the cell interior (Komoszyński and Wojtczak 1996). Both ecto-apyrases and endo-apyrases can be soluble or inserted in membranes (Wolf *et al.* 2007) and have the ability to hydrolyze both the  $\gamma$ - and  $\beta$ - phosphate of ATP and ADP

(Plesner 1995). Ecto-apyrases have diverse physiological functions, including the salvaging of extracellular nucleotides (Clark *et al.* 2014), modulation of ATP-mediated immunoresponses (Di 1998), protein glycosylation (Abeijon *et al.* 1993), and enhancement of soybean nodulation (Govindarajulu *et al.* 2009). Legume ecto-apyrases have been shown to be specific in their role in nodulation (McAlvin and Stacey 2005). The biochemical characteristics of plant endo-apyrases have been analyzed in *Pisum sativum* (Shibata *et al.* 2002) and *Solanum tuberosum* (Kettlun *et al.* 2005), though their physiological functions remain unknown.

*Arabidopsis thaliana* is a small flowering plant that is widely used as a model organism in plant biology. The advantages of *Arabidopsis thaliana* include its small genome, extensive genetic and physical mapping of all five of its chromosomes, a rapid life-cycle, which have facilitated its use in studies of the cellular and molecular biology of flowering plants. Because of these advantages, we chose *Arabidopsis thaliana* as a representative plant species to introduce the research on the apyrases.

### Types and functions of apyrases in *Arabidopsis thaliana*

To date, 18 members of the apyrase family have been discovered in plants. *Arabidopsis thaliana* contains 7 members (Steinebrunner *et al.* 2000), *Solanum tuberosum* contains 1 member (Handa and Guidotti 1996), leguminous plants contain 4 members, and *Mimosa* contains 6 members (Ishikawa *et al.* 1984, Ghosh *et al.* 1998).

In *Arabidopsis thaliana*, seven members of the apyrase family contain representatives from each clade. AtAPY1 and AtAPY2 are clustered into clade I. AtAPY3-6 are clustered into clade II, and AtAPY7 is located alone in clade III.

Among the seven members found in *Arabidopsis*, AtAPY1 and AtAPY2 have been extensively studied. Two apyrase genes (*AtAPY1* and *AtAPY2*) have been cloned and sequenced. The transcripts of *AtAPY1* and *AtAPY2* are widely distributed; however, the expression patterns are not identical. In roots, for example, the amount of mRNA of *AtAPY1* is greater than that of *AtAPY2*. Furthermore, AtAPY1 and AtAPY2 are 87 % identical in their amino acid sequences. Both contain four apyrase conserved regions (ACRs), an ATP-binding motif and a hydrophobic segment at the N-terminus. However, only AtAPY1 demonstrates a calmodulin-binding domain (Steinebrunner *et al.* 2000).

Both AtAPY1 and AtAPY2 have been shown to have numerous physiological functions, which are closely related to pollen development (Steinebrunner *et al.* 2003), vegetative growth, and stomata movements (Wu *et al.* 2007, Clark and Roux 2011a). Researchers use RNA interference to inhibit the expression of *AtAPY1* or *AtAPY2*, which results in structural changes of the cell wall

(Min *et al.* 2014). The data provides strong evidence to support the hypothesis that AtAPY1 and AtAPY2 function as plant endo-apyrases and are necessary to add saccharides to proteins or lipids. Nevertheless, their defined functional role as endo-apyrases would not necessarily preclude their roles as regulators of the ecto-ATP/ADP concentration *via* a secretory mechanism, as argued by Clark *et al.* (2014), based on immunochemical (Wu *et al.* 2007) and genetic data (Min *et al.* 2014) for the suppression of AtAPY1 and AtAPY2 causing an increase in extracellular ATP. Moreover, Yang *et al.* (2015) showed that the apyrases are associated with extracellular ATP and root skewing in *Arabidopsis*.

Besides *AtAPY1* and *AtAPY2*, the other five *Arabidopsis* apyrase genes (*AtAPY3-7*) have been less studied. *AtAPY3-5* occur as recurrent tandem duplications, sharing a 68 % identity (Chiu *et al.* 2015). All three are expressed during *Arabidopsis* development with *AtAPY3* being predominately found in the roots. Both *AtAPY4* and *AtAPY5* occur in the rosette leaves (Winter *et al.* 2007). From this, we may speculate that these enzymes have similar functions at different developmental stages. *AtAPY6* was confirmed to have a high expression in mature pollen, but knockout mutants of *AtAPY6* displayed a minor change in pollen exine pattern under scanning electron microscopy, which means *AtAPY6* plays a minor role in pollen development (Yang *et al.* 2013). In recent years, the molecular analysis of AtAPY7 has confirmed its ubiquitous expression in a range of *Arabidopsis* tissues and developmental stages, even though the molecular analysis of *AtAPY6* and *AtAPY7* mutants has indicated minor aberrations to the pollen exine (Chiu *et al.* 2015). Double

knockout mutants (*AtAPY6* and *AtAPY7*) show late-anther dehiscence, exine deformation, and low male fertility (Yang *et al.* 2013).

For several years, extracellular ATP has been proposed as a potential signalling molecule (Clark and Roux 2011a). Plant cells, for example, can release significant quantities of ATP into the extracellular matrix when they are

mechanically stimulated (Jeter *et al.* 2004), as occurs during wounding (Song and Roux 2006), growth (Kim *et al.* 2006), or stomatal opening (Clark and Roux 2011b). Meanwhile, Yang *et al.* (2015) have proved *AtAPY1* and *AtAPY2* can help regulate the concentration of extracellular ATP, which means the two apyrases can play indirect roles in signal transmission.

### Subcellular localization and main specific activities of the apyrases

Previously, only *AtAPY1* was believed to be localized in the Golgi instead of the extracellular space (Schiller *et al.* 2012). Later, both *AtAPY1* and *AtAPY2* were identified in plant Golgi proteomes (Parsons *et al.* 2012) and their localizations were confirmed by fluorescent protein tagging (Chiu *et al.* 2012, Schiller *et al.* 2012). According to current research, *AtAPY1*, 2, 4, 5, and 7 are localized in the *cis*-Golgi, and *AtAPY3* is localized in the endosome. Some evidence is for localization of *AtAPY6* in the endoplasmic reticulum.

Apyrase activities are strictly dependent upon the presence of divalent cations, with  $Mg^{2+}$  and  $Ca^{2+}$  being the most effective (Chiu *et al.* 2015). In addition, these enzymes are generally insensitive to specific inhibitors of P-, F-, and V-type ATPases and display a high specific activity (Komoszyński and Wojtczak 1996, Plesner 1995).

Apyrase family members have different substrate specificity (Chiu *et al.* 2015), and the clade I members, *AtAPY1* and *AtAPY2*, show a clear preference towards UDP and UDP/GDP. This may explain why knocking out either *AtAPY1* or *AtAPY2* affects UDPase/GDPase activity in microsomal preparations from *Arabidopsis*. Knocking out *AtAPY1* or *AtAPY2* resulted in a minor change in the galactose content of cell walls (Chiu *et al.* 2012). To be more specific, *AtAPY1* is an integral membrane protein that shows a clear preference towards UDP. The overexpression of *AtAPY2* can lower the sensitivity of *Arabidopsis* leaves to applied ATP, and *AtAPY1* and *AtAPY2* are essential for normal plant development. *AtAPY1* and *AtAPY2* play a key role in glycosylation, and are associated with extracellular ATP

(Yang *et al.* 2015). The clade II member, *AtAPY3* has a strong preference of nucleoside triphosphates (NTPs) but demonstrates significant activities toward ADP and GDP. Other members of the clade II apyrase family displayed an array of substrate preferences. *AtAPY4* shows only a slight affinity to cytosine triphosphate (CTP), *AtAPY5* has the highest catalytic activity for nucleoside diphosphate (NDP), which can turn NDP into NMP, and *AtAPY6* show a broad range of substrate activities toward all NTP and NDP substrates, which means it can catalyze both NTP and NDP. The clade III representative, *AtAPY7* shows no detectable NTPase or NDPase activity. In brief, all *AtAPY1-6* enzymes exhibit classic apyrase-like NTPase and/or NDPases activities, with no nucleoside monophosphate (NMP) activity, and *AtAPY7* does not show NTPase or NDPase activity.

What needs to be pointed out is that apyrases are divided into two main types according to their localization. *AtAPY1-7*, what we have introduced before, belong to endo-apyrases. The ecto-apyrases belong to those apyrases which are localized in the extracellular matrix. For *Arabidopsis thaliana*, several functions are related to the ecto-apyrases, including quenching of an ATP signal (Jeter *et al.* 2004, Tang *et al.* 2003), playing a role in toxin resistance (Thomas *et al.* 2000), and being involved in phosphate nutrition (Thomas *et al.* 1999, Song and Roux 2006). Most of these enzymes are ecto-phosphatases with their N-terminal (and sometimes their C-terminal) domains being anchored in the plasma membrane, with the rest of the protein positioned in the extracellular matrix (Komoszyński and Wojtczak 1996).

Table 1. Location and enzyme activities of apyrases in *Arabidopsis thaliana*.

Member	Location	NTPase or NDPase activities	Preference of substrates
<i>AtAPY1</i>	<i>cis</i> -Golgi	yes	UDP/GDP
<i>AtAPY2</i>	<i>cis</i> -Golgi	yes	UDP/GDP
<i>AtAPY3</i>	endosome	yes	NTP, ADP, GDP
<i>AtAPY4</i>	<i>cis</i> -Golgi	yes	slight affinity for CTP
<i>AtAPY5</i>	<i>cis</i> -Golgi	yes	NDP
<i>AtAPY6</i>	endoplasmic reticulum	yes	all NTP and NDP substrates
<i>AtAPY7</i>	<i>cis</i> -Golgi	none	none

## Progress and difficulties in apyrase research in *Arabidopsis thaliana*

Summary of progress:

- a) Seven members of the apyrases are discovered and classified;
- b) Each apyrase is subcellularly localized;
- c) The substrate preferences are determined;
- d) A preliminary understanding of the roles and functions of the apyrases are achieved, with a more complete understanding of the biochemical and subcellular characterization of AtAPY1 and AtAPY2.

The main difficulties in research of the apyrases are:

- a) Only small amounts of apyrases are available; in particular, AtAPY3-7;
- b) The new technologies are developed slowly, and researchers often need to develop specialized and innovative equipment and methods;
- c) Only a small number of researchers and laboratories are engaged in this field of research.

## Conclusions

Apyrases play multiple regulatory roles in the cellular activities of *Arabidopsis thaliana*. A better understanding of their different regulatory functions in different tissues may enable to use them in regulating plant growth and enhancing crop production. Although some technological

difficulties are present in research of the various members of the apyrase enzyme family, some progress in this area is evident. New technologies are developed to further characterize the apyrases, and the research involving *Arabidopsis thaliana* may prove to be especially useful.

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