Abscisic Acid, Microtubules and Phospholipase D -Solving a Cellular Bermuda Triangle

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Karlsruhe, im December 2023

Xuan Liu

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Abbreviations

2,4-D	2,4-dichloro phenoxy acetic acid
4-ATT	4-amino antipyrine
AA1	ANTAGONIST1
ABA	Abscisic acid
COLD-1	Chilling-tolerance Divergence 1
detyr	detyrosinated α-tubulin
DMG	3,3-dimethylglutaric acid
DTT	Dithiothreitol
GDP	Guanosine-5'-diphosphate
GFP	Green fluorescent protein
GTP	Guanosine-5'-triphosphate
HRP	Horseradish Peroxidase
MAP	Microtubule associated protein
MS	Murashige-Skoog
MT	Microtubule
PLD	Phospholipase D
PMSF	Phenylmethylsulfonyl fluoride
PTM	Posttranslational modification
RFP	Red fluorescent protein
TTC	Tubulin tyrosine carboxypeptidase
TTL	Tubulin tyrosine ligase
tyr	Tyrosinated α-tubulin

Zusammenfassung

Abscisinsäure (ABA) besitzt die Fähigkeit, das Pflanzenwachstum zu verhindern. Zudem bestehen Mikrotubuli aus den Untereinheiten α-Tubulin und β-Tubulin, welche als Ziel von postranslationalen Modifikationen (PTMs) dienen. Diese PTMs haben wiederum einen Effekt auf die Zellelongation und Zellteilung. Die häufigste PTM, der Detyrosinierung-Tyrosinierung-Zyklus steht in Beziehung zur Abspaltung eines Tyrosins des konservierten C-Terminals von α-Tubulin durch die Tubulin-Tyrosin-Carboxypeptidase (TTC) und die Religation des Tyrosins am C-Terminal durch die Tubulin-Tyrosin-Ligase (TTL). Um nähere Einblicke in die Dreiecksbeziehung zwischen ABA, Mikrotubuli und den Detyrosinierung-Tyrosinierung-Zyklus zu gewinnen, wurde eine stabile BY-2 Tabak Zelllinie mit überexprimierten *NtTUA3-GFP* (genannt NtTUA3) zur Markierung der Mikrotubuli generiert. Des Weiteren wurde eine doppeltransgene BY-2 Zelllinie mit der Co-Expression von expressing *NtTUA3-GFP/OsTTLL12-RFP* (genannt NtTUA3+OsTTL) generiert.

Die Phänotypanalyse in der Reis-Zelllinie AtTubB6 zeigte dass die Überexpression von MTs den Wachstum der Wurzeln und Koleoptilen der Reispflanze verzögerte. Ein exogener Einsatz von Abscisinsäure (ABA) und dessen Inhibitor ANTAGONIST1 (AA1) in Reis zeigte dass die Inhibition von Pflanzenwachstum mit dem detyrosinierten α-Tubulin korreliert.

Zusätzlich zu dem Effekt von ABA auf den Pflanzenwachstum wurde überraschenderweise herausgefunden, dass ABA die Kältestabilität der Mikrotubuli vorübergehend induziert durch exogenen Einsatz von ABA und AA1 auf die Zelllinie NtTUA3. Diese Phänotypen wurden begleitet von der vorübergehend erhöhten Enzymaktivität von Phospholipase D (PLD).

Die Analyse von detyrosinierten und tyrosinierten α-Tubulin in der Zelllinie

NtTUA3 oder in NtTUA3+OsTTL durch Western-Blot führte zum Fazit dass ABA nicht nur einen regulatorischen Effekt auf detyrosinierte α-Tubulin hat, sondern auch abhängig vom Protein TTL12 ist. Detyrosiniertes α-Tubulin, welche durch PLD Aktivierung, die durch 1% n-Butanol induziert wurden, zeigten dass ABA detyrosiniertes α-Tubulin auf eine andere Art und Weise induzieren gegenüber n-Butanol. Durch die Messung der der Aktivität von PLD und dem Level von detyrosinierten α-Tubulin nach Behandlung mit Pertussis-Toxin (PTX), einem G-Protein Inhibitor, gefolgt von ABA sowie die Behandlung mit Mastoparan deuten darauf hin, dass der Effekt von ABA und PLD auf detyrosinierten α-Tubulin parallel verlaufen.

Abstract

Abscisic acid (ABA) has property of preventing plant growth. Moreover, microtubules are composed of α-tubulin and β-tubulin that are the target of post-translational modifications (PTMs) that have effect on controlling cell elongation and cell division. The detyrosination/tyrosination cycle as the most common PTMs is related to the removal of the conserved C-terminal of α-tubulin by a tubulin tyrosine carboxypeptidase (TTC) and relegation of this tubulin by a tubulin tyrosine ligase (TTL). To get the insight into the triangular relationship in ABA, microtubules and detyrosination/tyrosination cycle, a rice tubulin marker line overexpressing *AtTuB6-GFP* (referred as AtTubB6), a tobacco BY-2 microtubule marker cell line stably overexpressing *NtTUA3-GFP* (referred as NtTUA3 line) as well as the double transgenic BY-2 cell line co-expressing *NtTUA3-GFP/OsTTLL12-RFP* (referred as NtTUA3+OsTTL line) were generated.

The phenotype analysis in rice AtTUB6 line demonstrated that overexpression of MTs retarded the growth of roots and coleoptiles of rice plants. Exogenous application of ABA and its inhibitor ANTAGONIST1 (AA1) in rice AtTubB6 provided evidence that the inhibition of plants growth by ABA is correlated with the detyrosinated α-tubulin.

In addition to the effect of ABA on plants growth, it was found, unexpectedly, that ABA also can transiently induce cold stability of microtubules through exogenous application of ABA and AA1 on a NtTUA3 line. These phenotypes were accompanied by the transient increase of phospholipase D (PLD) enzyme activity.

The analysis of detyrosinated /tyrosinated α-tubulin by western blot in NtTUA3 line or in NtTUA3+OsTTL line gave us such a conclusion that the effect of ABA

on detyrosinated α-tubulin not only was regulated by ABA but also was dependent on TTLL12 protein. The detyrosinated α-tubulin caused by PLD activation that induce by 1% n-butanol has shown that ABA induced detyrosinated α-tubulin in a manner distinct from n-butanol. Through detecting the PLD activity and detyrosinated α-tubulin level after pretreatment with pertussis toxin (PTX), a G-protein inhibitor, followed by ABA, as well as mastoparan (Mas7) treatment suggested that the effect of ABA and PLD on detyrosinated α-tubulin was in parallel.

1. Introduction

Cold stress is a major abiotic stress, which also limits the growth and development of plants (Wang *et al*., 2017). One of the earliest molecular responses of cells to cold stress is the rapid disassembly of microtubules (Shelanski *et al*., 1973). Therefore, microtubules as potential targets for cold sensitivity have been discussed for a long time. Moreover, ABA can lead to microtubular cold stability (Wang *et al*., 2001). In addition to ABA, the post-translational modification of α-tubulin and β-tubulin is related to the difference of microtubule stability and structure, so it is expected to be a candidate for such molecules to describe events. Therefore, we would like to test the relationship between ABA and detyrosination/tyrosination cycle under cold stress. Both of phospholipase D (PLD) and rice tubulin tyrosine ligase like 12 protein（TTLL12）as the signal hubs can induce detyrosinated α-tubulin (Zhang *et al*., 2021; Zhang *et al*., 2022), so, we also prefer to detect the role of PLD and TTLL12 in response to ABA in cold condition.

Apart from a role of ABA for microtubular stability in cold stress, ABA as the stress hormone has the property of preventing plant growth. In addition, detyrosination/tyrosination cycle as the most common post-translational modification can control cell elongation and cell division (Zhang *et al*., 2021). However, what is the relationship between ABA and detyrosination/tyrosination cycle to response plant growth? Is it unclear now. To get information relevant for plants development in response to ABA and detyrosination/tyrosination cycle, we use rice plants as a system. This will involve stable transformation of rice, as well as phenotyping in rice.

1.1 cold stress

1.1.1 The impact of low temperatures on crop plants

In the world, the one of biggest challenges we face is climate change including low temperatures, which can limit the growth and development of many crop plants to cause significant damage to the economy (Repo, *et al*., 2008). For example, the freezing that occurred in California has caused about $7 billion in economic losses to oranges, avocados and other crops (Smith *et al*., 2021). Due to the response to low temperatures, cold stress is divided into two classifications. One is the freezing tolerance with sub-zero (<0°C) causing irreversible membrane damage. The other one is the chilling tolerance with a temperature between 1-15°C. The plants which can endure freezing tolerance, evolve from temperature regions. And when they are put in the temperature with slightly over 0°C resulting in cold acclimation (Juurakko *et al*., 2021). As a consequence, it prepares plants for low temperatures. The Table 1 shows that various plant classifications depending on the response of these plants to different temperatures (Table 1).

Table 1. The plants are classified into freezing or chilling tolerance depending on their capacity of low-temperature tolerance. Table source: Juurakko, 2021.

Species	Classification	Capacity (°C)	Refs.
Triticum aestivum (wheat)	Freezing tolerant	-20°C	Thomashow, 1998
Solanum tuberosum (potato)	Chilling tolerant	-3°C	Chen et al., 1976
Solanum lycopersicum (tomato)	Chilling sensitive	10°C	Saltveit and Morris, 1990
Oryza sativa (rice)	Chilling sensitive	17°C	Shakiba et al., 2017
Arabidopsis thaliana (thale cress)	Freezing tolerant	-6 to -11°C (depending on accession)	Kaplan et al., 2004; Hannah et al., 2006
Brachypodium distachyon (purple or stiff brome)	Freezing tolerant	-12°C	Mayer et al., 2020
Lolium perenne (perennial ryegrass)	Freezing tolerant	-13°C	Thomas and James, 1993
Zea mays (maize)	Chilling sensitive	10°C	Rodríguez et al., 2014
Nicotiana tabacum (tobacco)	Chilling sensitive	2°C	Zhao et al., 2009
Rhododendron catawbiense (purple ivy)	Freezing tolerant	-54°C	Wei et al., 2005

1.2 Microtubule

Plants cannot migrate when facing cold stress (Wang *et al*., 2017). To survive, microtubules play an important role in acting as a susceptor to receive the input, and then convey these signals to downstream reactions rapidly (Wang *et al*., 2020). Therefore, microtubules as potential targets for cold sensitivity have been discussed for a long time.

1.2.1 Early development of microtubules

Cytoskeleton, which was discovered in the 20th century, are composed of actin filaments and microtubules (Green, 1962). For the concept of microtubules, Porter and Ledbetter first provided evidence by searching for the cellulose deposition according to Paul Green's prediction (Slautterback, 1963; Ledbetter & Porter, 1963). They thought that a microtubule is a fine structure under the surface of protoplasts, with a small tubular structure between 230 and 270A in diameter, which can reflect the orientation of cellulose microfibers in adjacent cell walls. After that, it soon gave rise to the concept of tubulin, which has the property of binding to a chemical, named colchicine with microtubules-destabilizing (Weisenberg *et al*.,1968). However, with the development of studies in the microtubules, in the 1980s, it identified that microtubules are dynamic and unstable depending on the time-lapse (Mitchison *et al*., 1984; Mitchison *et al*., 1986). In recent years, many scientists agreed with the fact, that the dynamic properties of microtubules are linked to the microtubules associated proteins (MAPs) which can influence plant growth by controlling cell morphology, cell division, substance transport, energy conversion, information transmission, cell differentiation, cell elongation and cell polarity (Marc *et al*., 1998; Nick, 2012; Nick, 2013; Parrotta *et al*., 2014).

Since Ledbetter and Porter discovered microtubules in plant cells in 1963 (REF),

great progress has been made in their research. Cyclic nucleotides and the sulfhydryl state of microtubules can change the balance of cell regulatory activity. So, to understand it better, we put the introduction of microtubules in advance.

1.2.2 The basic structure of microtubule

Microtubules, an essential element of the cytoskeleton, comprise α- and β-tubulin dimmers and also can maintain dynamic balance by regulating the shrinking phase and regrowth phase by themselves (Zhang *et al*., 2021). In most eukaryotic cells, the complex functions with modulating cell division and expansion require microtubules to have a hollow cylinder structure including 13 protofilaments (Hashimoto, 2015; Ledbetter & Porter, 1964). Like the description in the Fig 1(a). In brief, α- and β- tubulin dimmers generate protofilaments by stacking longitudinally with each other. Then, the protofilaments formed from the above step will be aligned in parallel, resulting in the generation of cylindrical polar structures of microtubules. The α-tubulin and β- tubulin which are exposed ends represent minus end and end, respectively. In addition, due to the connection of α- and β- tubulin monomers of a protofilament with the one of adjacent protofilaments respectively, it forms a structure named B-type lattice. The crucial question is how the microtubules disassemble. The most important step is the generation of polar protofilaments of microtubules, where the E-site of the GTP-binding site of β- tubulin which acts as a plus-end can hydrolyze GTP to GDP, whereas the N-site of the GTP binding site of α-tubulin doesn’t have this function of hydrolyzing GTP to GDP. Because of these reactions, a GTP cap is formed in the growing microtubule resulting in the asymmetrical and self-arranged heterodimers of α- and β-tubulin to promote polymerization (Fig. 1b).

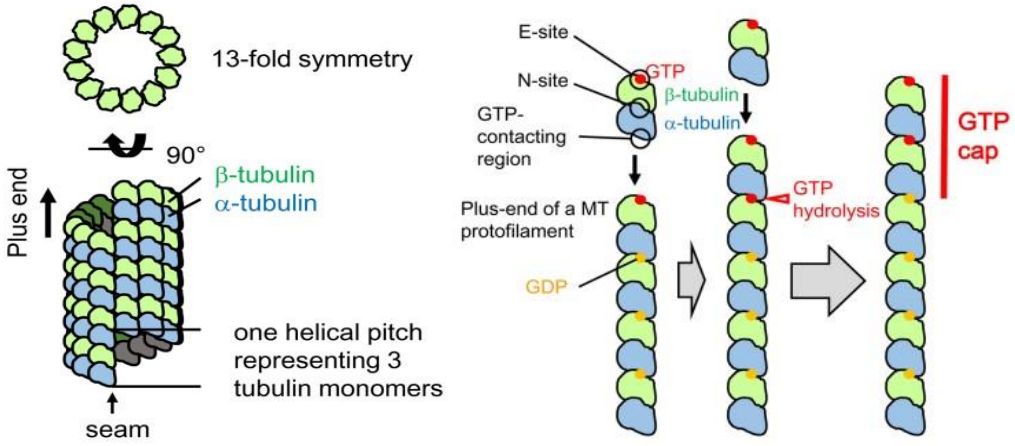


Fig. 1. (a) microtubule structure. Thirteen parallel protofilaments assemble into microtubules with a hollow cylindrical structure generated by longitudinally stacking α- and β- tubulin heterodimers. (b) The model of hydrolyzation of GTP to GDP in the microtubules. α-tubulin connect the N-site which can't hydrolyze GTP, whereas the E-site of β-tubulin has the function of hydrolyzation from GTP to GDP. Image source: Hashimoto, 2015.

Microtubules are highly dynamic in nature. In fact, the protofilaments composed of GTP- tubulin is straight, forming a stable MT lattice that is easy to polymerize, while the protofilaments of GDP- tubulin is curved, forming an unstable MT lattice, which is easy to depolymerize through the weak transverse interaction between the profilaments. Therefore, MT polymerization and GTP hydrolysis are mechanically linked, resulting in the unique metastable behavior of MTS (Hashimoto, 2015). There is a model called GTP-Cap to descript the growth of microtubules. Like in the Fig. 2, When GTP- tubulin occupied the plus end, MT continued to grow. but the loss of this ability caused by the overspeed hydrolysis and random dissociation of GTP- tubulin exposed the unstable core of GDP-tubulin, resulting in outward stripping and rapid depolymerization of GDP-precursors.

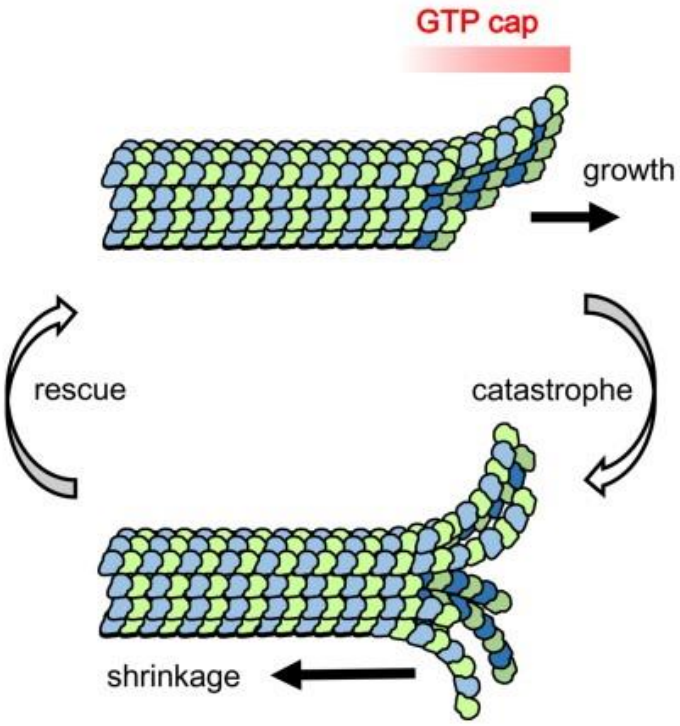


Fig. 2 The model of dynamic instability of microtubules. Image source: Hashimoto, 2015.

1.2.3 The role of microtubules in cold stress

Different observations support the role of MTs in plant cold sensation: in winter wheat, the rapid disassembly of cortical MTs has been proved to be a necessary and sufficient condition for initiating cold adaptation, followed by stable MTs bundle formation (Abdrakhamanova *et al*., 2003). Under low-temperature conditions, the decrease of membrane fluidity occurs unevenly, resulting in local asymmetry, which can induce a minus force originating from the borderline of fluid and less fluid patches (Knight *et al*., 1991; Wang *et al*., 2020). After that, microtubules can collect this minus force and amplify it to induce downstream reactions. Like in Fig. 3, the membrane fluidity is first reduced by cold stress to lead to minus mechanical forces, which can be integrated by microtubules to activate calcium channels along with the stimulation of Chilling-tolerance Divergence 1 (COLD 1), which was a QTL was cloned by Chinese Academy of Sciences (Ma *et al*., 2015). Because of the activation of COLD1, the enzyme of

PLD is induced to finish the following series of reactions (Ruelland *et al*., 2002; Munnik *et al*., 1995)

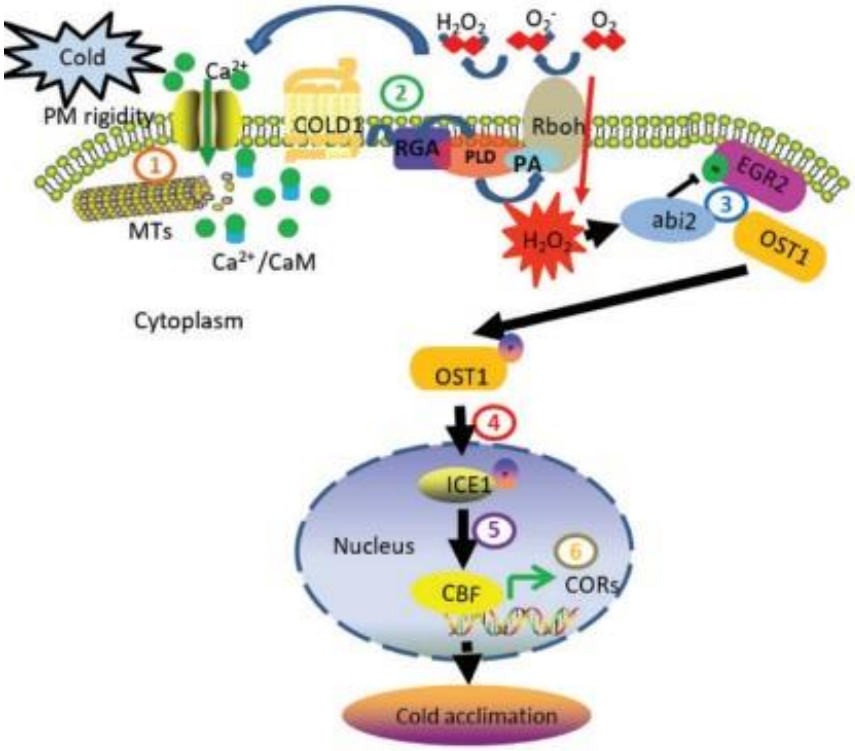


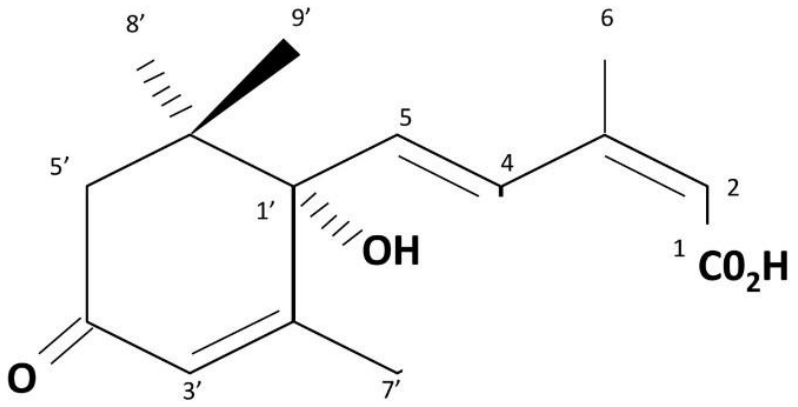
Fig. 3. Work model of cold signalling. Under cold stress, membrane rigidity, microtubules and calcium channels as the centre of the cold susceptor system deliver the signal to the facilitator named COLD1 resulting in the activation of PLD enzyme activity to induce further reactions. Image source: Wang, 2020.

1.3 Abscisic acid

In addition to microtubules, when plants face abiotic stresses, such as salt stress, cold stress and osmotic stress, will lead to changes in cellular hormone levels. Subsequent studies revealed that abscisic acid (ABA), a plant hormone, has been shown to play an important role in the adaptation of plants to different abiotic stresses (Xiong & Zhu, 2003). Therefore, we discussed the role of ABA in cold stress and also in other abiotic stresses in the following sections.

1.3.1 introduction of ABA

It has been identified that ABA, a sesquiterpenoid ($C_{15}H_{20}O_4$), includes 15-carbon (C15) (Fig. 4). Discovered In the early 1960s, scientists identified a compound with the capacity to accelerate abscission, then it was named “abscisin II”. After that, this compound was renamed abscisic acid, together with a dormancy-inducing factor, which was called “dormin”, isolated by researchers in the UK from sycamore leaves (Nambara & Marion-Poll, 2005; Wasilewska *et al*., 2008; Cutler *et al*., 2010).



Fid. 4. Structure of phytohormone abscisic acid S- (+) -ABA.

1.3.2 the function of ABA in cold stress

When plants are exposed to low temperatures, many cellular functions will be affected, such as the change of endogenous hormone levels, the decrease of membrane fluidity, and the destruction of membrane lipid composition (Lyons & Raison, 1970; Sharma *et al*., 2005). Among them, changes in the level of endogenous hormone, such as ABA, has been proved to improve the adaptation of plants to low temperature (Verslues & Zhu, 2005; Fujii *et al*., 2009; Kim *et al*., 2016). Previous studies have shown that in many plants, cold stress is accompanied by an increase in the level of endogenous ABA resulting in improvement of the cold resistance of plants (Mantyla *et al*., 1995; Kumar *et al*.,

2008; Kim *et al*., 2016). In presence of cold stress, the gene expression is dependent or independent on ABA pathway also will be activated. For example, in Arabidopsis, the expression levels of ABA responsive transcription factor, ABF1 and ABF4, are induced under cold stress (Choi *et al*., 2000).

1.3.3 the function of ABA in other abiotic stresses

Drought stress is also one of the main adverse environmental factors for plant growth and development, which regulates many aspects of plant growth and development. ABA accumulation is the one of the most important ways for plants to enhance drought tolerance. When plants are facing to drought stress, The stomatal closure can be induced by ABA and the water from roots can be absorbed because of ABA, thus maintaining water balance in plants. In addition, it also has shown that drought stress can trigger ABA biosynthesis in maize root cells (Lü *et al*., 2007).

In addition to drought stress, the plant growth also is affected seriously by salt stress leading to metabolic process and photosynthetic efficiency. The main reason for formation of salt stress is because of the accumulation of Na^+ and Cl^- (Tester *et al*., 2003). However, some studies have shown that the application of exogenous ABA decreased the sodium concentration in sorghum (Amzallag *et al*., 1990) and kidney bean (Khadri *et al*., 2007) or their transport to the shoot, resulting in salt tolerance adaptation, while exogenous ABA increased the Karmapan Na^+ ratio of rice, which was related to the improvement of salt tolerance (Bohra *et al*., 1995).

Therefore, ABA is not only an important regulator of plant growth (Barrero *et al*., 2005; Fujii *et al*., 2009), but also can act as a stress hormone to induce stress tolerance of plants (Lü *et al*., 2007).

1.3.4 the relationship between ABA and microtubules

Microtubules play important roles in response to abiotic stress. Under abiotic stress, the elevated ABA level improves plant stress resistance (Rezaul *et al*., 2019). Therefore, it is worth to discuss the relationship between microtubules and ABA.

When plants are under drought stress, the hormone ABA induces stomatal closure to avoid excessive water loss. During the stomatal closure induced by ABA, the microtubule skeleton is depolymerized rapidly (Wang *et al*., 2023). In brief, in this study, when the ABA level increases, microtubule-binding protein SPIRAL1 (SPR1) can be phosphorylated by OPEN STOMATA 1 (OST1), the core component of the ABA signalling pathway. Phosphorylated SPR1 dissociates from microtubules, promotes microtubule depolymerization and regulates ABA-induced stomatal closure (Fig. 5).

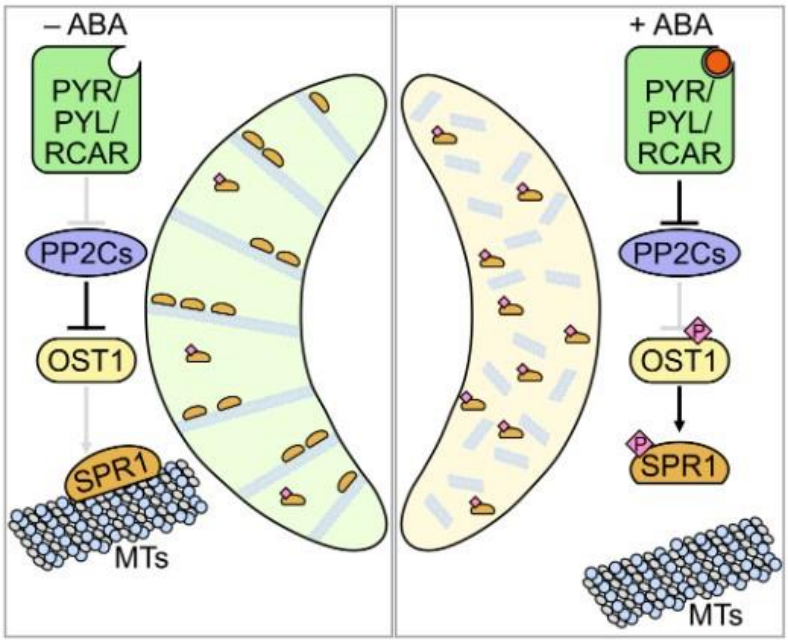


Fig. 5. OST1-SPR1 regulates the molecular model of microtubule depolymerization and stomatal closure induced by ABA. In the process of microtubule depolymerization induced

by ABA and stomatal closure, the serine at position 6 of SPR1 was phosphorylated by OST1. Phosphorylated SPR1 dissociates from microtubules, promotes microtubule depolymerization, and then regulates stomatal closure induced by ABA. Image source: Wang, 2023.

Apart from the effect of ABA on stomatal closure when plants facing to drought stress, there is also evidence to support the point that ABA can also assist to induce cold resistance (Boussiba *et al*. 1975, Rikin *et al*. 1976, Chen *et al*. 1983). The cold resistance of plants is often accompanied with the elevated content of ABA, which has effect on various kinds of plants, such as *Acer negundo* (Irving 1969), cucumber seedlings (Rikin & Richmond 1976), and apple seedlings (Holubowicz & Boe 1969). In fact, the factors that improve the cold resistance of plants will lead to the improvement of the cold stability of microtubules (Sakiyama &Shibaoka, 1990; Kerr & Carter, 1990, Pihakaski-Maunsbach & Puhakainen, 1995). On the basis of this finding, it has shown that the ABA prevents the cold-induced disassembly of microtubules to increase plants resistance. For example, in cotton, the application of exogenous ABA can not only prevent chilling injury in the presence of antimicrotubular drugs but also inhibit the destruction of MTs under cold stress (Rikin *et al*., 1980; Rikin *et al*., 1983). At the same time, this effect also is proved in winter wheat (Wang *et al*., 2001).

1.4 PLD signalling pathway

Cold stress can decrease membrane fluidity unevenly, which can result in the production of minus forces to trigger the reorganization of microtubules to improve plant adaptation (Wang *et al*., 2020). Now, there is the question: how does the membrane connect with the microtubules?

Phospholipase D is identified from a 90-kDa polypeptide isolated from tobacco as a membrane junction protein which can hydrolyze structural phospholipids, such as phosphatidylcholine, to produce phosphatidic acid (PA) and free choline (Munnik, 2001; Wang, 2001; Wang *et al.*, 2002; Marc *et al.*, 1996). Based on this principle, some studies also showed that PLD can act as a bridge to connect the membrane and MTs. One hypothesis that supports this view is that PLD when PLD hydrolyses phospholipids, it forms a covalent bond with the phospholipids in the membrane as well and the choline group will be removed. If the intermediate is stable *in vivo* for some time, PLD and microtubules will continue to adhere to the membrane. On the contrary, once PLD is activated. The phosphatidyl group will be transferred to the water (Dhonukshe *et al.*, 2003; Munnik & Musgrave, 2001). Therefore, from the above steps of PLD activation, it can get two results, one is that activation of PLD results in microtubules reorientation. Another is that PA is produced to trigger the reactions downstream (Fig. 6).

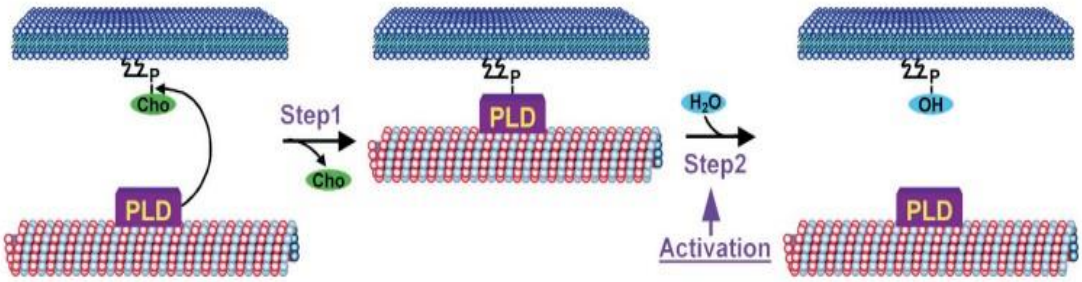


Fig. 6. The model for plant microtubule reorganization. The whole process is divided into two steps. First step: The choline group leaves PLD and its associated microtubule to covalently connect to the phosphatidyl group. Second step: The PLD-microtubules complex is activated to transfer the phosphatidyl group to water. Image source: Dhonukshe, 2003.

As the signal hub, the function of the PLD enzyme depends on the activation of

heterotrimeric G-proteins which are present in the plants and also can activate effector enzymes and induce physiological responses (Zhao *et al*., 2004). By applying with pertussis toxin, an inhibitor of G-protein, and Mas7, an activator of G-protein, it revealed that MT reorganization was dependent on PLD activity activation (Dhonukshe *et al*., 2003; Keen *et al*., 2022).

1.5 Detyrosinaton/tyrosination cycle

It has to be identified that PLD, a 90-kDa protein firstly isolated from tobacco membranes, is thought to be linked to detyrosinated α-tubbulin (Zhang *et al*., 2022), which is one of the most common post-translational modifications (Zhang *et al*., 2021). The change of cyclic detyrosination-tyrosination as the most prominent PTM has been shown to correlate with the reorientation of cortical MTs, a key regulator of axial cell expansion in plants (Wiesler *et al*., 2002). Almost all eukaryotic α-tubulins contain a C-terminal tyrosine that can be cleaved off by a tubulin-tyrosine carboxypeptidase (TTC) yielding a detyrosinated α-tubulin (MacRae, 1997). Like in Fig 7, The antagonist of TTC is a tubulin tyrosine ligase (TTL), which can relegate a tyrosine to detyrosinated tubulin (Zhang *et al*., 2021). Under normal circumstances, the tyrosinated form is dominant (Gundersen *et al*., 1987). However, for example, in tumour cells, the abundance of tyrosinated α-tubulin decreases, and the inhibition of TTL activity promotes tumour invasion (Lafanechere *et al*., 1998). There is only one tyrosine difference in the structure, but the difference is significant. In recent years, inhibition of α-tubulin detyrosination has been found to recruit the plant-specific binding of class XIV-kinesin KCH to cortical microtubules, which is involved in the structure and sensory function of the microtubule cytoskeleton (Schneider *et al*., 2015).

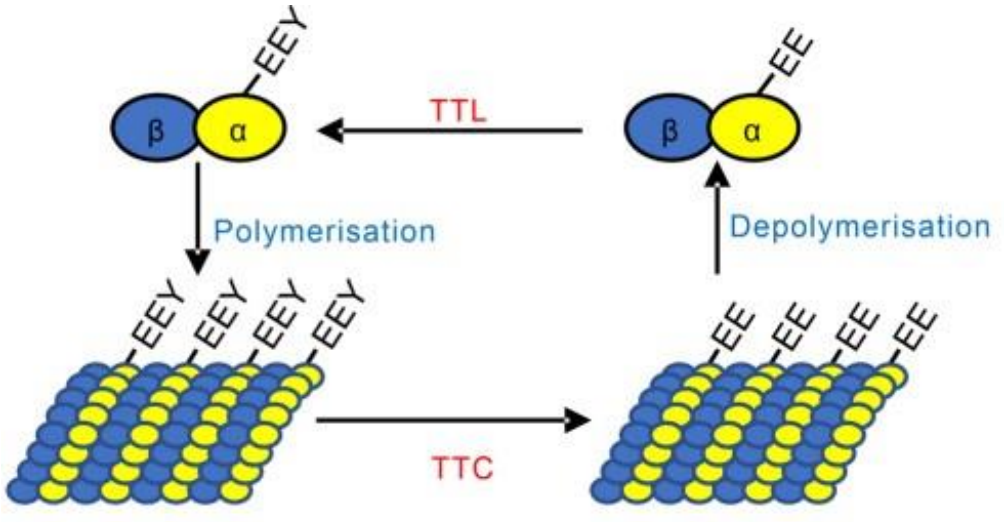


Fig. 7. The model for detyrosination and tyrosination cycle of α‐tubulin.

1.6 Significance of the project

The project attempts to understand the role of the microtubule-phospholipase D signalling hub for cold stress in ABA. Microtubules, as central regulators of plant growth and development, not only play an important role in the response of plants to abiotic stress but also are related to the cell wall and plasma membrane as sensory centres for perceptual stimulation and activation of downstream targets. This may enhance the adaptability of plants to abiotic stresses (Nick, 2013). Although the role of microtubules in stress is becoming progressively evident, the regulation of microtubule reorganization in response to stress conditions is still far from clear. Therefore, the project will contribute to a deeper understanding of the mechanisms by which cortical microtubules are involved in stress signal transmission and processing.

Cold stress is a major limitation for plants in temperate regions. Because plants cannot avoid abiotic stress by migration, they have obtained a variety of stress resistance mechanisms in the process of evolution (Wang *et al*., 2017). ABA

acts as a stress hormone, which is possible to improve cold tolerance by activation of a so-called cold hardening. Therefore, we would like to test if this signalling underlying this cold hardening involves changes in the dynamics of cortical microtubules and the activity of phospholipase D. The comprehensive understanding of these mechanisms is important to improve agricultural production on a biotechnological base.

1.7 Scope and objectives of the project

The project wants to address the function of the interaction between microtubule and phospholipase D as a central hub for cold stress signalling under ABA conditions. From this scope, the following objectives are derived:

1. How can we follow the response of ABA to microtubules in cold stress in vivo?

Since pretreatment with ABA before using antimicrotubular drugs can prevent chilling injury (Rikin *et al*. 1980, Rikin *et al*. 1983). There is a question: how does the microtubule change in cold stress in the presence of ABA? To test this hypothesis, a GFP-based tubulin marker line is generated in tobacco BY-2 cells, by stable expression of the marker gene *TUA3*. In the following, ABA as well as AA1, an ABA inhibitor, were used to treat the BY-2 cell line overexpressing *NtTUA3-GFP* to observe the response of microtubules in presence of cols stress.

2. How does ABA interact with modulations of PLD activity under cold stress?

PLD as a signal hub can be activated by cold stress. It is possible to be a bridge to connect membrane and microtubules, so, to test whether the application of ABA affects the activity of PLD enzyme, we measured the PLD enzyme activity in the BY-2 cell line overexpressing *NtTUA3-GFP* in the different time points of

ABA treatment or along with cold stress.

3. How do tubulin modifications interact with ABA treatment?

Since the affinity-chromatography approach had revealed phospholipase D as a binding partner for detyrosinated α- tubulin . From the above step, one question is whether ABA affects the distribution of detyrosinated/tyrosinated α-tubulin. To investigate this assumption, the level of detyrosinated/tyrosinated α-tubulin was detected by western blot using antibody DM1A and ATT, respectively, under the ABA treatment of the BY-2 cell line overexpressing *NtTUA3-GFP*. If so, the further question is, does ABA activate PLD first or detyrosinated/tyrosinated α-tubulin? To address this question, we used pertussis toxin (PTX) and mastoparan (Mas7) , an inhibitor and an activator respectively of G-protein which can stimulate PLD activity, to treat the BY-2 cell line overexpressing *NtTUA3-GFP* to detect how detyrosinated/tyrosinated α-tubulin level change.

4. Is rice tubulin tyrosine ligase-like 12 protein (OsTTLL12) affected by the application of ABA?

Previous work in our lab revealed that TTLL12 protein would increase detyrosinated α-tubulin levels (Zhang *et al*., 2021). Therefore, to link the function of OsTTLL12 with ABA, a double transgenic line stably overexpressing *OsTTLL12-RFP* in a microtubule marker line *NtTUA3-GFP* tobacco BY-2 cell line (Zhang *et al*., 2022) was produced. Then, ABA was applied in this line to dissect the detyrosinated α-tubulin level by western blot.

2. Materials and Methods

2.1 standard chemicals, reagents and equipment

All standard chemicals we used came from Roth (Karlsruhe, Germany), Sigma-Aldrich (parent of company of Sigma, Aldrich and Fluka, Steinheim, Germany) as well as Duchefa (Haarlem, the Netherlands), unless stated otherwise.

All antibodies for western blotting were purchased from Sigma-Aldrich, and all materials were used in the western blot came from Roth (Karlsruhe, Germany). The enzymes for digesting plasmid and T4 ligase were purchased from New England Biolabs (NEB, Frankfurt, Germany).

In experiments, the water for preparing all solutions and media came from an Ultra Clean UV plus system (SG, Barsbüttel, Germany). A Seradest SD2800 filter was used to produce deionized water which can be used to rice cultivation. The various equipment we used in this study is mentioned in the respective chapter.

2.2 BY-2 cell lines

2.2.1 cell cultivation

All the suspension tobacco culture (Nicotiana tabacum L. cv Bright Yellow-2, BY-2) including the cell line expressing the fluorescent tubulin marker *NtTUA3-GFP* (termed TUA3), and a line, expressing, in addition, the Tubulin Tyrosine Ligase Like 12 from rice in fusion with RFP (termed TTLL+TUA3) were used and cultivated in the liquid Murashige-Skoog (MS) medium containing 4.3 g/L MS salts (Duchefa, Haarlem, The Netherlands), supplementing with 30 g/L sucrose, 200 mg/L K_2HPO_4, 100 mg/L Myo-inositol, 1 mg/L thiamine, and 0.2

mg/L 2,4-dichloro phenoxy acetic acid (2,4-D), at least, the pH should be adjusted to 5.8. To sustain selective stringency, the respective antibiotics were added, 50 mg/L kanamycin in case of TUA3, or 30 mg/L hygromycin and 50 mg/L kanamycin in case of TTL+TUA3. Cells were subcultured by transfer of 1.5 mL cell suspension into 30 mL of fresh medium in a 100 mL Erlenmeyer flask. The subculture interval was biweekly. Cells were incubated in the dark at 27°C under constant agitation on an orbital shaker at 150 rpm. The following is the respective cell line we used in this study.

2.2.2 Cell line used in the experiment

In the whole experiments, we used three different tobacco BY-2 cell lines (Table 2.1).

(1). Non-transformed Bright Yellow 2 (BY-2) Wild type

(2). NtTuA3-GFP: this line is expressing tobacco tubulin α3 (NtTuA3) as GFP fusion (GFP fused to the N-terminus to let the C-terminus, where the MAPs bind unmasked) under control of the strong and constitutive Cauliflower Mosaic Virus 35S promoter (CaMV 35S)

(3). NtTUA3+OsTTL: we transformed the pH7WGR2-OsTTL (N-terminal fusion of the red fluorescentprotein, the constitutive CaMV 35S promoter, hygromycin resistance;accession number of OsTTL: LOC_Os03g08140) into BY-2 cell line overexpressing *NtTUA3-GFP*, then the double tobacco BY-2 cell line expressing *OsTTL-RFP* and microtubule marker *NtTUA3-GFP* was generated as described in Klotz & Nick, (2012).

Table 2.1 Transformed and non-transformed BY-2 cell lines used in this study

Name	Genotype	Application	Source
WT	Wild Type (WT) Nicotiana tabacum L.cv Bright Yellow 2	Control	
TUA3	NtTUA3-GFP cell line, Overexpression NtTUA3	Microtubule marker line	Berghöfer *et al*., 2009
TTL+TUA3	Overexpression OsTTL-RFP in NtTUA3-GFP BY-2 cell line	Signaling analysis	Zhang *et al*., 2022

2.2.3 Measuring Phospholipase D activity

In plant cells, the activation of PLD correlates is followed by the reorganisation of cortical microtubules. Therefore, we measured PLD activity *in vivo* as described by Mao *et al*. (2007). In brief, soluble proteins were extracted according to Zhang *et al*. (2022) by spinning down cell debris with 1000 g for 15 min, transferring the supernatant to a fresh tube and centrifuging a second time with 15,000 g for 30 min to remove organelles. The clear supernatant was then spun down with 105,000 g for 1 h to yield a sediment of microsomal membranes, which was then dissolved in 100 mM 3,3-dimethylglutaric acid (DMG) at pH 6.5 for the extraction of PLD. Enzyme activity was determined by a colourimetric method by Huang *et al.* (1997) using 20 µL of the microsomal preparation in a total volume of 200 µL assay mixture (Table 2.2). The reaction

was incubated at 30°C for 30 min and then transferred into the boiling water for 15 min to denature the proteins. After that, the result was visualised by adding 800 µl of chromogenic solution (45 mM Tris-HCl, pH 8.0), 0.8 units choline oxidase, 2.4 units Horseradish Peroxidase (HRP), 0.24 mg oxidized 4-amino antipyrine (4-ATT), and 0.16 mg phenol) incubating at 30°C for 90 min. Before taking the readout, 1 mL of Tris-HCl (45 mM, pH 8.0) was supplemented with 0.2 % w/v Triton X-100. To exclude any potential turbidity which might perturb the readout, the solution was filtered through a 0.22-µm syringe filter before measuring A_{500}. Data represent mean and standard error from three biological replications.

Table 2.2 the composition of basic assay mixture for PLD measurement

Component	Concentration (mM)
$MgCl_2$	25
$CaCl_2$	5
3,3-dimethylglutaric acid buffer	5
linoleic acid	1
phosphatidyl-choline	1

2.2.4 Different drug and cold treatments

2.2.4.1 ABA and cold treatments in BY-2 cell lines

The TUA3 suspension cells collected on day 7 after sub-cultivation were transferred into 2-mL reaction tubes (Eppendorf, Hamburg), placed in a bath of ice water (0°C) and incubated in the dark on a horizontal shaker. To monitor the effect of ABA on the cold response of microtubules, 2 mL of TUA3 cells were first pre-treated with 50 µM ABA (Duchefa, Harlem, The Netherlands) for defined time intervals (1 h, 2 h, and 3 h) at 25°C, before exposure to ice water

for 1 h. Cells were observed by microscopy immediately afterwards using a pre-cooled slide and coverslip to delay re-warming. To address the role of endogenous ABA during the cold response, the inhibitor ANTAGONIST 1 (AA1, Life Chemicals, Niagara, Canada), was administered in 500 µM at 25°C for 1 h to TUA3 cells. This inhibitor blocks the binding pocket of the ABA receptor and, thus, disrupts, ABA signalling (Ye *et al.*, 2017). Subsequently, these cells were subjected to cold stress for 1 h before assessing the state of microtubule depolymerisation. During all treatments and incubations, the cells were kept in the dark while shaking at 150 rpm. Parallel to microscopical inspection, a second set of each sample was shock-frozen in liquid nitrogen to be used later for measuring the activity of Phospholipase D. In a second set of experiments, both TUA3 and TTL+TUA3 cells were treated with 50 µM ABA for defined time intervals (up to 5 h) at 25°C in the dark to be shock-frozen.

2.2.4.2 n-butanol treatment

n-butanol, which can bind the product of PLD, phosphatidic acids (PA), thus intercepting PLD signalling (Munk *et al.*, 1995). Strains TUA3 and TTL+TUA3 were pretreated with 1% *n*-butanol for 1 h, before adding 50 µM ABA and sampling at 1 h, 2 h, and 3 h after addition of ABA. A parallel set of experiments followed the effect of *n*-butanol alone, sampling at 0 h, 1 h, 2 h, and 3 h.

2.2.4.3 Mastoparan and Pertussis Toxin Treatments

To address whether activation of a G-protein could mimic the effect of ABA, we treated for 1 h with the Mastoparan analogue Mas 7 at 5 µM, comparing the response to a sample without Mastoparan. To test, whether G-protein activity is needed for the response to ABA, we used the inhibitor Pertussis Toxin (PTX) at 10 µg/mL for 1 h, before the addition of 50 µM ABA, and sampled at 1 h, 2 h,

and 3 h after addition of ABA. This experiment was conducted in strain TUA3 only.

2.2.5 Monitoring and quantifying microtubule responses

The response of microtubules to the different treatments was followed in the tubulin marker strain TUA3 by spinning disc confocal microscopy as described in Wang and Nick (2017) using an AxioObserver Z1 microscope (Zeiss, Jena, Germany) with a cooled digital CCD camera (AxioCamMRm; Zeiss), exciting the GFP signal with the 488-nm line of an Argon-Krypton laser (Zeiss, Jena, Germany). Images were collected into Z-stacks and orthogonal projections were constructed with the ZEN Blue software (Zeiss, Jena, Germany) and then exported as TIFF files for further analysis. Microtubule integrity was quantified as described in Wang and Nick (2017) using a probing line with a width of 8 pixels to buffer against background noise and collecting four intensity profiles in equal spacing parallel to the long cell axis of the cell. The principle of this quantification is to determine the steepness of the intensity peaks (reflecting microtubules) by determining the first derivative of the peaks. When microtubules disassemble, this is reflected by filling up the troughs between the peaks, such that steepness is progressively dissipating. Data represent three biological replications with 20 individual cells per replication.

2.3 Rice Plants

2.3.1 Rice lines and cultivation

For getting the rice tubulin marker line, we digested the plasmid named pH7WGF2 (Nakamura *et al*., 2004; Hohenberger *et al*., 2011) from enzyme sites BamHI to SacI, resulting in the fragment including *AtTuB6* and GFP

sequence. After that, we digested plasmid pCAMBIA5300 in the same enzyme sites and transferred this fragment we got from the above step into it using T4 DNA ligase. Then, the resulting construct was used to be transferred into the rice plant background of wildtype Kitakka. The rice tubulin marker line was cultivated as described in this study (Tang *et al*. 2020). In brief, first, the seeds overexpressing *AtTubB6-GFP* were washed with 70% ethanol 1min, then followed with washing 3 times of double distilled water. For deep surface sterilization, we put rice seeds into the 12% sodium hypochlorite solution for 20 minutes before washing them with double distilled water four times. Last, we have sown these sterilized seeds in the Magnata box including 2g/500mL of Phytoagar and 0.172g/500mL of Murashige and Skoog Basal Salt Mixture (MS) or double-layer filter papers for different experimental purposes.

2.3.2 DNA extraction

The leaves harvested from rice plants overexpressing *AtTubB6-GFP* first were ground to powder in a 2mL safe-lock tube. For extraction, 1.5% boiled CTAB buffer was added to this safe tube which would incubate for 1h at 65°C. then, we added 630 µl of Chloroform: isoamyl alcohol solution (24:1) in the sample obtained from the above step and shook horizontally for 15 min at 75 rpm at room temperature. To get the supernatant containing the nucleic acids, we centrifuged it at 15,000rpm for 10 minutes. After that, to get the precipitated nucleic acids, we added 2/3 v/v ice-cold isopropanol (e.g. supernatant 700 µl, isopropanol 450 µl) in the supernatant and centrifuged at 15,000rpm for 10 minutes in room temperature. Then, we used 70% ethanol to wash the precipitated nucleic acids and remove the supernatant. After washing, we dried it in a vacuum centrifuge for 15 minutes and added 50 µl 1/10 TE buffer containing 5 µg RNase A for RNA digestion for dissolving DNA. In the last step, keep the sample for 2 hours at room temperature before freezing at 20°C.

2.3.3 phenotyping of rice lines

After testing that the rice tubulin marker line was pure, we sown them in the Magnata box for 7 days as described in the above step, then moved them into the MS liquid medium (0.172g/500 ml) for 21 days to observe the plant height and leaf inclination in the rice tubulin marker line and wildtype, respectively.

To determine the growth of roots and coleoptiles, we imaged at day 5 after sowing in the double layers papers as described above. The software Image J was used to measure the length of primary roots and coleoptiles. Three biological replicates were analyzed.

2.3.4 ABA and AA1 treatment

For ABA treatment, the seeds were sown on the double filter paper soaked with 1 μM ABA or 500 μM AA1 solution and cultivated in the dark condition. They were added in the dim green safelight (λ_{max} 550 nm, 20 m W m^{-2}) to avoid the induction of plant photoreceptor phytochrome. The roots and coleoptiles were harvested on day 5 after sowing to do the western blot.

2.4 Molecular Biology Methods

2.4.1 RNA extraction and cDNA synthesis

The leaves of rice TubB6 line for 21 days was used to extract RNA using the NucleoZOL total RNA extraction kit according to the instructions of the manufacturer. In brief, the tissues are lysed and homogenized in NucleoZOL reagent containing guanidinium thiocyanate and phenol. Contamina.ng molecules such as DNA, polysaccharides, and proteins are precipitated by the

adding water and then removed by centrifuge. RNA can be reconstituted using RNase-free water. After that, we tested the RNA quality obtained from the above step through electrophoresis on a 1% agarose gel. The cDNA was synthesized using the M-MuLV cDNA Synthesis Kit (New England Biolabs) according to the instructions of the manufacturer. The amount of RNA as a template for this step was 1 μg.

2.4.2 Quantitative real-time PCR (qRT-PCR) analysis

The Bio-Rad CFX machine was used to perform real-time PCR and the gene expression level was calculated with the $2^{-\Delta Ct}$ method and normalized against internal reference gene. We put the reaction system and cycler conditions in Table 2.3 and Table 2.4.

Table 2.3 qPCR mix per reaction

Component	Amount (μ L)
5X Go Taq Puffer buffer	4
5 mM each dNTP mix	0.4
10μM primer forward	0.4
10μM primer Reverse	0.4
0.5U/μl GoTaq Polymerase (Promega)	0.1
SybrGreen	0.95
cDNA template (1:10)	1
Mgcl2 (50mM)	1
Dd H2O	11.75
Final volume	20

Table 2.4 Cycling conditions of qRT-PCR

Temperature (℃)	Time
95	3 min
95	15 s
60	40 s

2.4.3 primers used in this work

Table 2.5 The sequences of forward and reverse primers used in the study

Name	Sequence
OsUBQ5 Fw	ACCACTTCGACCGCCACTACT
OsUBQ5 Rv	ACGCCTAAGCCTGCTGGTT
AtTubB6 Fw	TGGCAAGATGAGCACAAAAG
AtTubB6 Rv	GCAGGTCACTGGATTTTGGT
Hygromycin Fw	GCCTGACCTATTGCATCTCC
Hygromycin Rv	TACACAGCCATCGGTCCAGA

2.5 Protein extraction and Western blot

2.5.1 Protein extraction from rice and tobacco BY-2 cells

For biochemical analysis of protein, we harvested roots and coleoptiles of rice plants at day 5 after sowing under green light to avoid the induction of plant photoreceptor phytochrome. Then, the roots and coleoptiles we got from rice were ground to powder which will transfer to a 2mL tube and added the same volume of cold extraction buffer (Table. 2.6). Then, we mixed them vigorously. After centrifuging 30min at 13500 rpm, the particles containing cell wall debris

and other insoluble residues were removed, and the soluble proteins in the supernatant were collected for Western blots.

Proteins extraction wea prepared from 7 days cells of the TUA3 and TUA3+TTL lines, respectively. After harvesting, then they were ground into powder under liquid nitrogen condition, following by the same volume cold extraction buffer (Table. 2.6). Through centrifuging at 13500rpm for 30min at 4°C, insoluble tissue debris was removed and the soluble protein was collected for western blot.

Table 2.6 Standard buffer for protein extraction

Component	Concentration (mM)
MES	25
EGTA	5
MgCl2·6H2O	5
DTT	1
PMSF	1
KOH	Adjusts pH to 6.9

2.5.2 Protein concentration measurement

The protein concentration was determined by a Bradford assay (50 mg Coomassie Brilliant Blue 250 in 25 ml of ethanol: phosphoric acid: water 1:17:3). First, we prepared a 10mg/mL BSA stock solution, after that, to get the standard curve, we diluted a 2mg/mL BSA solution out of the 10mg/mL stock solution to make different concentration gradient (Table 2.7) and measured them at 595 nm. Second, we mixed 10 uL of each sample with 1mL Bradford solution and stained for 15 min before measurement at 595 nm. The calculation of protein concentration according to this formula: A 595 nm'* factor (in µg) / volume of

the sample (in µl).

Table. 2.7 Different standard curve concentrations

BSA concentration in mg/ml	2 mg/ml BSA solution in µl	Water in µl
0	0	100
0,2	10	90
0,4	20	80
0,6	30	70
0,8	40	60
1	50	50
1,2	60	40
1,4	70	30
1,6	80	20
1,8	90	10
2,0	100	0

2.5.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The extracted protein was mixed with 3x loading buffer (table. 2.8) and then denatured at 95°C for 5 min. the total protein was loaded, adjusting the volume to give equal concentrations of total proteins per lane. The gel we used for loading was 10% (w/v) SDS-polyacrylamide gels (Table. 2.9). After 90 minutes of running at a constant 25 mA in a mini gel tank (Life Technologies, ThermoFisher Scientific) with electrophoresis buffers. The samples were run in triplicate, one set was stained with Coomassie Brilliant Blue staining to verify the equal loading of lanes, and the other two sets were used for Western blotting to detect detyrosinated or tyrosinated a--tubulin, respectively.

Table. 2.8 Compositions of 3X loading buffer

Substrate	Components	Concentration
3X loading buffer	Glycerol	30% (W/V)
	DTT	300mM
	SDS	6% (W/V)
	Stacking gel buffer	48% (W/V)
	Bromphenolblue	0.05% (W/V)
	Milli Q water	To 10mL

Table 2.9 Compositions of 10% SDS-PAGE

Substrate	Components	Separation gel (10%)	Stacking gel (4%)
30% Acrylamide/Bis-solution	30 % (w/v) Acrylamide and 0.8 % (w/v) Bisacrylamid	8.2 mL	1.3mL
Separation gel buffer	1.5 M Tris-HCl (pH=8.8) and 0.6 % (w/v) SDS	6.2mL	
Stacking gel buffer	0.5 M Tris-HCl (pH=6.8) and 0.6% (w/v) SDS		2.3 mL
ddH2O		10.3 mL	
TEMED	40 % (w/v) N;N;N、 ,N、 ,-Tetramethylethylendiamin in milli Q water	108 µL	52.8 µL
APS	10 % (w/v) ammonium sulphate in milli Q water	215.9 µL	105.7µL

2.5.4 western blot and signal development

We used the method named semi-dry western blotting to transfer proteins to a polyvinylidene (PVDF). For this purpose, first, we cut 4 pieces of filter paper and a piece of PVDF membrane to the same size as the SDS-gel that the stacking gel was removed. The transfer buffer and methanol were used to soak the filter paper for 5min and the PVDF membrane for 1 minute before western blot. Beginning from the anode, like showing in the Fig 2.1, in order to avoid air bubbles, we placed two sheets of filter paper on the anode (positive), followed by the membrane, the gel, two sheets of filter paper, and finally the cathode (negative).

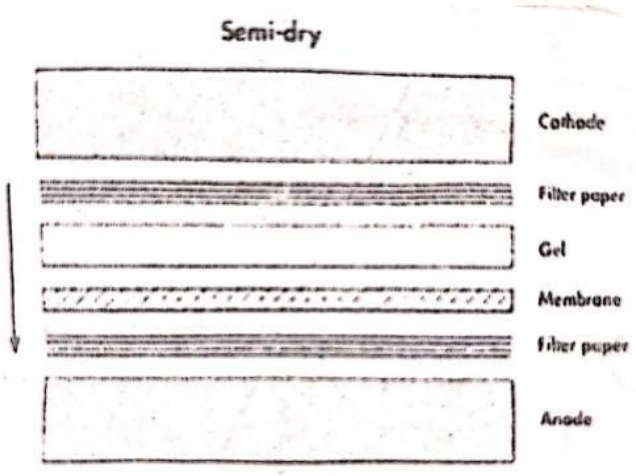


Fig. 2.1 The model of Semi-dry for transferring membrane.

The blot was run 1h at a voltage of 25. After protein transfer, the PVDF membrane was washed two times with TBS buffer (Table. 2.10) for 10min at room temperature. After washing, to avoid unspecific binding sites in the membrane, we incubated for 1h in blocking buffer (5% milk buffer (w/v) in TBS buffer), followed by two times washing using TBST buffer (Table. 2.10). Then, the PVDF membrane was incubated in the primary antibody (1:3000 in TBS buffer) overnight at 4 °C. The primary antibody was removed by washing twice for 10min each time in TBST buffer at room temperature followed by washing for 10min in TBS buffer also in the room temperature. Subsequently, the

membrane was incubated in the secondary antibody for 1h (1:30000 in TBS buffer). After that, the secondary antibody was removed by rinsing four times in TBST buffer for 10min.

For signal development, 2mL NBT/BCIP (Sigma, Germany) was used to detect the target protein bands. The signals were quantified by measuring the integrated density as described by Schneider *et al.* (2015) of each band on the Western blot by ImageJ (https://imagej.nih.gov/ij). For quantification, the relative percentage of tyrosinated or detyrosinated a-tubulin, respectively was determined over the entire signal for both tubulin pools.

Table 2.10 Solution required for western blot and signal development

Labelling	Content	Concentration
Transfer buffer	Glycine	1,4% (w/v)
	Tris	1,2% (w/v)
	Methanol	20% (v/v)
TBS buffer (pH 7.4)	Tris/HCl	20mM
	NaCl	150mM
	Triton X-100	1% (v/v)
TBST buffer (pH 7.4)	Tris/HCl	20mM
	NaCl	150mM
	Triton X-100	1% (v/v)
	Tween-20	0.1% (v/v)

2.5.5 Antibodies

ATT is a monoclonal antibody, which is derived from the hybridoma produced by the fusion of mouse myeloma cells and splenocytes from an immunized

mouse. It can detect tyrosinated α-tubulin specifically due to its epitope contains the following amino acid: VEGEGEEEGEEY (Kreis 1987).

DM1A is also a monoclonal antibody and can detected detyrosinated α-tubulin. The epitope consists of the amino acids 424-430 (DMAALEK, Breitling & Little, 1986) within the protein.

In western blot, for signal detecting, a secondary antibody against mouse produced in goat was used. This antibody is coupled to alkaline phosphatase and stains on polyvinylidene fluoride (PVDF) membrane by phosphorylating its substrates nitrobluetetrazolium (NBT) and 5-Bromo-4-chloro-3-indolyl.

3. Results

3.1 Function of ABA in response to cold stress

3.1.1 ABA can increase the cold stability of cortical microtubules.

The induction of cellular resistance to cold stress is often linked with an accumulation of ABA, often accompanied by an elevated cold tolerance of microtubules (Wang & Nick, 2001). We, therefore, addressed the role of ABA in the cold response of microtubules in tobacco BY-2 cells, expressing the tubulin marker TuA3-GFP. In the absence of cold stress, we did not detect any significant effect of 50 µM ABA (administered for 60 min) compared to control (Fig. 3.1 A, B). Likewise, treatment with 500 µM of AA1, for 60 min, an inhibitor of ABA signalling (Ye *et al.*, 2017), did not cause any alteration of microtubules (Fig. 3.1 C). In contrast, the cold response of microtubules was modulated when the ABA status was manipulated. When control cells were subjected to 0°C for 60 min, the transverse arrays of microtubule bundles seen, if cells were kept at 27°C had mostly vanished, with individual and thinner microtubules remaining on the background of a cytosolic tubulin-GFP signal (Fig. 3.1 D). This microtubule elimination was mitigated by a pre-treatment with 50 µM ABA for 60 min (Fig. 3.1 E). In contrast, a pre-treatment with 500 µM AA1 accentuated the cold-induced elimination of microtubules (Fig. 3.1 F).

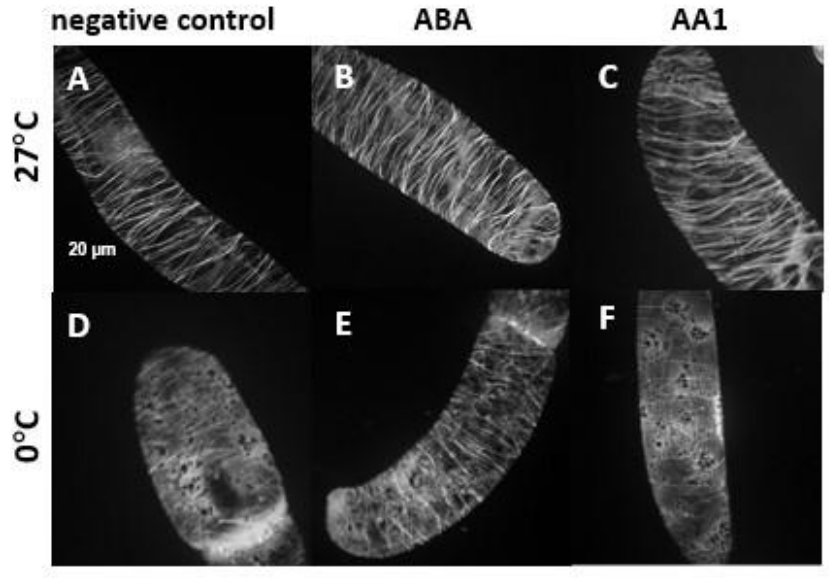


Fig.3.1 The response of cortical microtubules to modulation of ABA status and cold stress in cells expressing the *NtTUA3-GFP* marker. Representative cells either treated at 27°C (**A-C**) or at 0°C (**D-F**) either without treatment (**A, D**), after treatment with 50 µM of abscisic acid (**B, E**), or the abscisic acid antagonist AA1 (**C, F**) for 60 min.

To test the significance of these differences, we quantified microtubule integrity under these conditions (Fig. 3.2). We observed that a cold treatment of 60 min 0°C reduced microtubule integrity by around 40% (Fig. 3.2 A). Pre-treatment by ABA reduced this loss in integrity in response to a subsequent cold treatment to 23%. This is to be seen in the fact that ABA alone did not alter microtubule integrity. When cells were treated with the ABA inhibitor AA1, this did not affect microtubule integrity, there was even a mild, albeit not significant, increase of around 10% (Fig. 3.2 B). However, the subsequent response to the cold treatment was accentuated significantly (55% decrease of integrity) over the response seen for cold stress alone (40% decrease of integrity). Thus, ABA causes the stabilisation of microtubules against cold stress, and disruption of ABA signalling enhances the elimination of microtubules by cold.

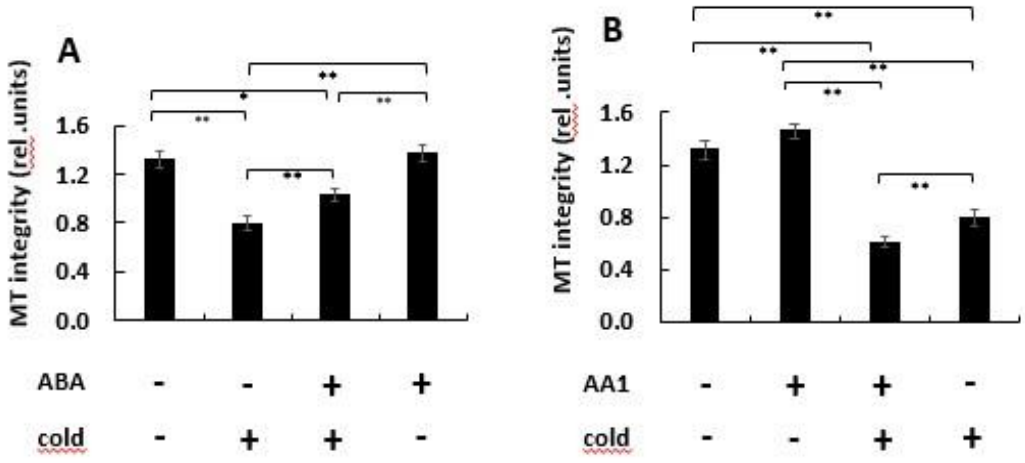


Fig. 3.2 Quantification of the response of cortical microtubules to modulation of ABA status and cold stress in cells expressing the *NtTUA3-GFP* marker. Microtubule integrity was scored after 60 min of incubation either in the absence of cold stress at 27°C (-) or at 0°C (+) following a pre-treatment with either 50 µM ABA for 60 min (**A**) or with 500 µM of AA1, a blocker for ABA signalling for 60 min (**B**) as compared to the control in the aspect of the respective agent. Data represent mean and SE from three biological replications with a sample of 20 individual cells per replication. Differences were tested for significance with a Student's t-test for paired data with * indicating significant differences at P <0.05 and ** at P <0.01.

3.1.2 The cold stability induced by ABA occurs in the early stage

The cold resistance of microtubules was conspicuously increased by a pretreatment of ABA 1h, a well-known inducer of cold hardiness. Then, we want to test whether prolonged ABA pre-treatment might promote this stabilizing effect. We found that cortical MTs were significantly affected under pre-treatment with ABA 120 min and 180 min in the presence of cold stress, resulting in a reduction of MT disassembly by 34% and 32%, which was a

significant difference compared to control (Fig. 3.3). There was less effective, although still leading to a slight stabilization as compared to cold stress without ABA-pre-treatment.

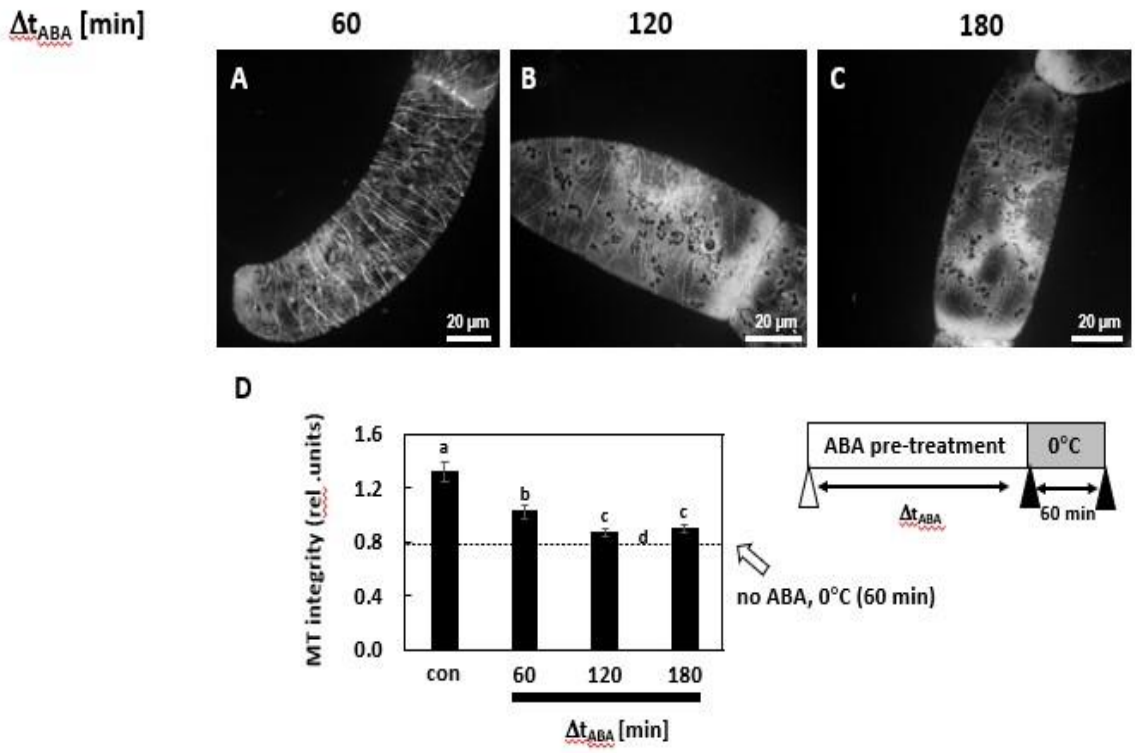


Fig. 3.3 Time dependence of the stabilising effect of ABA pre-treatment on microtubules. A-C representative cells were pre-treated for 60 min (**A**), 120 min (**B**), and 180 min (**C**) with 50 µM ABA and then challenged by cold stress (60 min, 0°C) in cells expressing the *NtTUA3-GFP* marker. **D** Microtubule integrity either in the absence of cold stress at 27°C (con) or at 0°C for 60 min following ABA pre-treatment for the indicated time intervals. Data represent mean and SE from three biological replications with a sample of 20 individual cells per replication. Differences were tested for significance with a Duncan test based on the Least Square Difference. Different letters mean differences at a significance level of $P < 0.01$.

3.1.3 ABA and cold can induce the activity of Phospholipase D.

PLD has been described as a hub for stress signalling including low temperature and has been identified as a necessary component for the microtubular response to cold (Wang & Nick, 2017). We, therefore, measured the enzymatic activity of PLD using a colourimetric assay (Huang *et al.*, 1997) in response to ABA (60 min, 50 µM) and response to cold stress (60 min, 0°C). Both factors increased PLD activity significantly, by almost 50%, over the resting level (Fig. 3.4 A). The combination of ABA and cold stress did not yield an additive effect, the resulting stimulation of PLD activity was only slightly (and not significantly) higher than that seen for each factor alone. To assess, whether the stimulation in response to cold stress was dependent on ABA, we blocked ABA signaling by AA1, an inhibitor of ABA (Fig. 3.4 B). While AA1 administered in the absence of cold stress did not cause any change in PLD activity, it did not interfere with the stimulation caused by cold stress. Thus, while ABA can stimulate PLD activity, ABA signalling seems to be dispensable for the stimulation of this enzyme by cold.

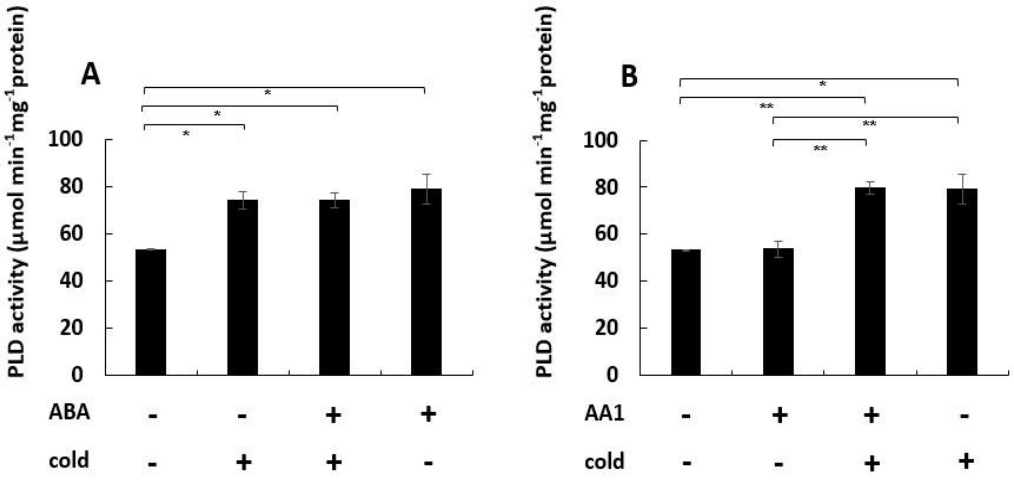


Fig. 3.4 Response of Phospholipase D activity to modulation of ABA status and cold stress in cells expressing the *NtTUA3-GFP* marker. Specific activity was measured after 60 min of incubation either in the absence of cold stress at 27°C (-) or at 0°C (+) following a pre-

treatment with either 50 µM ABA for 60 min (**A**) or with 500 µM of AA1, a blocker for ABA signalling for 60 min (**B**) as compared to the control in the aspect of the respective agent. Data represent mean and SE from three biological replications. Differences were tested for significance with a Student's t-test for paired data with * indicating significant differences at P <0.05 and ** at P <0.01.

3.1.4 The function of ABA inducing PLD activity is transient

From the above steps, we have concluded that the response of microtubules to ABA was transient, where the function of ABA just worked in the first 60 minutes, after 120min and 180 min, it disappeared gradually. Therefore, to understand the temporal dynamics of enzyme stimulation, we measured PLD activity over increasing duration of ABA treatment (Fig. 3.5) and found that this effect was transient. At 2 h after the addition of ABA, the stimulation had already dropped to around 25% above the resting level, and after 3 h, it had already fully dissipated.

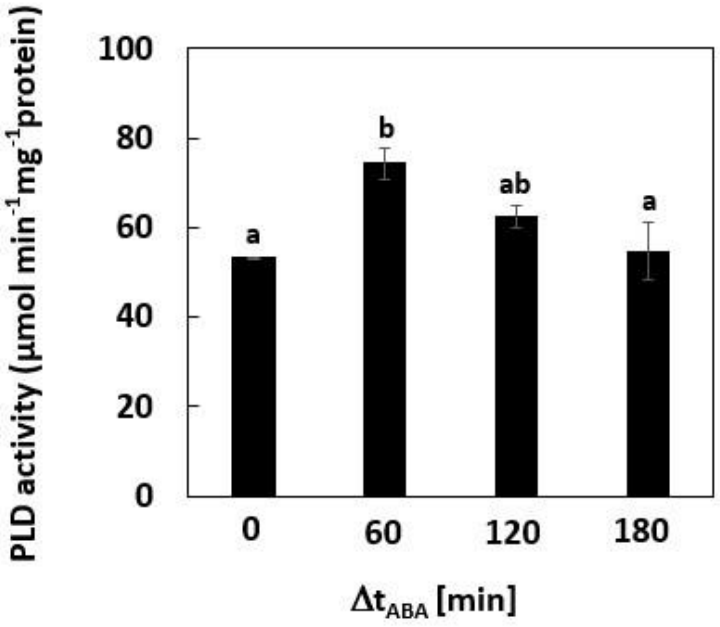


Fig. 3.5. Time dependence of the stimulation of PLD activity by ABA. Cells were treated with 50 µM of ABA at 27°C. Data represent mean and SE from three biological replications.

Differences were tested for significance with a Duncan test based on the Least Square Difference. Different letters mean differences at a significance level of P <0.01.

3.1.5 ABA can transiently increase the pool of detyrosinated tubulin.

It has been verified that activation of PLD which binds to detyrosinated a-tubulin can render MTs more stable. we wondered whether this stabilization might be linked to a reduced turnover of microtubules. Since the lifetime of microtubules correlates with post-translational modifications such as detyrosination of a-tubulin, we addressed the ABA response of the tyrosinated versus the detyrosinated tubulin pools using monoclonal antibodies differentiating between the two forms of a-tubulin (Fig. 3.6). The antibody raised against tyrosinated a-tubulin recognised a characteristic double band (Fig. 3.6 A) at around 55 kDa, whereby the upper band corresponds to the tyrosinated form, while the lower band is caused by a cross-reaction with the detyrosinated form that is sometimes but not reliably observed (Wiesler *et al.*, 2002). In addition, a higher band of above 80 kDa was detected that corresponds to the fusion of tobacco α3 tubulin and GFP present in this marker line (Zhang *et al.*, 2022). While the band presumably reported tyrosinated tubulin was persistent in response to ABA, the band stemming from the cross-reaction with detyrosinated tubulin was significantly fading out, especially for prolonged treatment with ABA. Since the detection of this band is coming from a cross-reaction and, therefore, cannot be used as a reliable readout for detyrosinated tubulin, we probed the same samples with a monoclonal antibody specially targeted to detyrosinated a-tubulin. This antibody did not show any cross-reaction with tyrosinated tubulin, such that here, only two bands were visible (the lower band of the tubulin duplex, and the band at around 80 kDa originating

from the GFP fusion of tubulin). Here, the signal for detyrosinated tubulin first increased significantly at 1 h after the addition of ABA but subsequently decreased at 3 h and almost vanished. To quantify this effect, control and ABA-treated extracts were run side by side in numerous replications, such that the bands could be quantified relative to each other using the respective control (incubation in the absence of ABA) as reference. This quantification (Fig. 3.6 B) showed that the pool of tyrosinated tubulin was first not very responsive but remained comparable to the control over 2 h. However, after 3h of ABA treatment, it dropped significantly by around 40%. In contrast, the detyrosinated pool was strongly responsive with a transient induction of 60% after 1 h of ABA treatment and a subsequent decline to around 80% below the control level, when the ABA treatment was prolonged to 3 h.

Thus, ABA can induce a transient stabilization of microtubules against cold (Fig. 3.3), transient stimulation of PLD activity (Fig. 3.5), and a transient increase of the detyrosinated pool of a-tubulin (Fig. 3.6 B). All three phenomena evoked by ABA show their peak at 60 min and subsequently disappear.

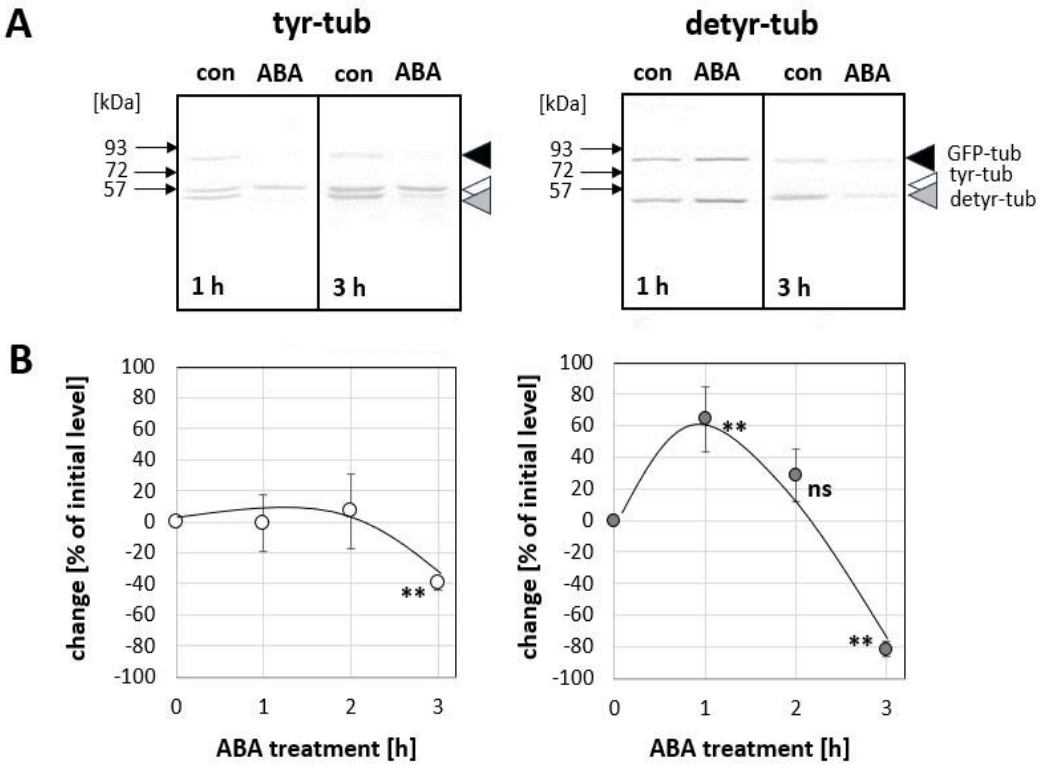


Fig. 3.6. Response in the post-translational modification of a-tubulin to ABA in cells expressing the *NtTUA3-GFP* marker. (**A**) Representative Western blots after probing with monoclonal antibodies against tyrosinated a-tubulin (tyr-tub) and detyrosinated a-tubulin (detyr-tub) for 1 h or 3 h of treatment with 50 µM ABA as compared to control cells incubated without ABA (con). The white arrowhead indicates the bona-fide tyrosinated form, the grey arrowhead the bona-fide detyrosinated form of a-tubulin, and the black arrowhead the bona-fide GFP fusion of tobacco a3 tubulin. (**B**) Quantification of the relative change (in %) of the respective tubulin form in response to ABA as compared to the abundance seen in the control run in the adjacent lane of the blot. Data represent mean and standard errors from three independent extractions. Differences were tested for significance with a Student's t-test for paired data with ** indicating significant differences at P <0.01 and ns non-significance at P >0.05.

3.1.6 ABA interaction with TTLL12 protein regulates tubulin modification

Our previous work (Zhang *et al*., 2021), it had identified that PLD activity was regulated by a rice tubulin tyrosine ligase like 12 (TTLL12), to verify this hypothesis, we followed the response of ABA on detyrosinated/tyrosinated α-tubulin in the double transgenic cell line TUA3+TTL12. We conducted a western blot analysis probing for tyrosinated and detyrosinated α-tubulin by using the monoclonal antibodies ATT and DM1A, respectively (Appendix Fig. S3). To quantify this effect clearly, the bands obtained from the western blot were quantified relative to each other using the respective control as a reference. The tyrosinated α-tubulin showed no significant differences after 1h and 2h of ABA treatments either in TUA3 line (without TTLL12) or TUA3+TTLL line (with TTLL12), whereas after 3h of ABA treatment, the tyrosinated α-tubulin level dropped and increased significantly by around 30% and 20% in TUA3 line and TUA3+TTLL line, respectively (Fig 3.7 A, C). In contrast, there was no difference in the detyrosinated α-tubulin level after 1h and 2h of ABA treatments in the TUA3+TTLL line, compared to control. After 3h of ABA treatment, it has a decline to around 20% below the control level (Fig 3.7 B, D). However, in our expectation, the response of ABA on detyrosinated a-tubulin should be elevated after ABA treatment. We couldn't forget that the system has to deal with a high level of TTLL12 already before ABA application and an induction of detyrosinated α-tubulin would therefore be reduced.

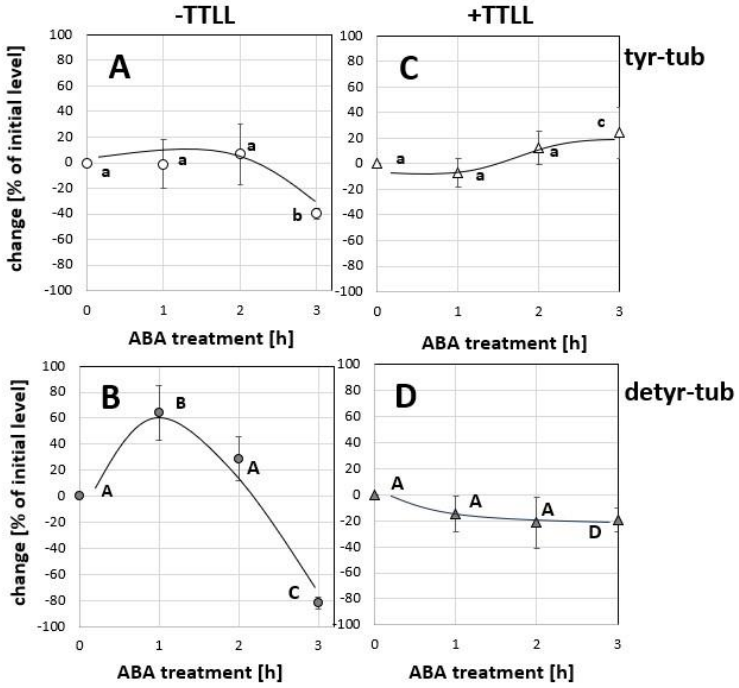


Fig. 3.7 Response in the post-translational modification of a-tubulin to ABA in cells expressing the *NtTUA3-GFP* marker either in the absence (**A, B**) or presence (**C, D**) of rice TTLL. Quantification of the relative change (in %) of the respective tubulin form in response to ABA as compared to the abundance seen in the control run in the adjacent lane of the blot over time of treatment. Data represent mean and standard errors from three independent extractions. Statistical differences were tested by the Tukey LSD test, different letters indicate that the respective data point belongs to a different population at $P<0.05$.

3.1.7 The response of detyr- tub to ABA is independent on the n-butanol pathway

N-butanol consumes PA, leading to a lower activity of MAP65-1. And the C-terminus of α-tubulin is the binding site for MAP65. The OsTTLL12 can compete with the C-terminal binding site of α-tubulin with MAP65-1. So, the application of n-butanol would induce OsTTLL12 protein function to produce

more detyrosinated α-tubulin (Zhang *et al*., 2022). Since the property of n-butanol, it was used to test whether the above hypothesis was feasible. we quantified the resulting signals (Appendix Fig. S4) from wastern blot. After n-butanol treatments, the tyrosinated α-tubulin level didn't show any difference significantly. Interestingly, the detyrosinated α-tubulin level just induced significantly and reached by around 150% over the control level after 2h of n-butanol treatment (Fig. 3.8 E, F).

Then, the TUA3 cell line was pretreated with 1% n-butanol 1h at 27°C, followed by ABA 1h, 2h and 3h. After that, a western blot was used to detect detyrsinated/tyrosinated α-tubulin by probing by ATT (directed to tyrosinated α-tubulin) and DM1A (directed to detyrosinated α-tubulin). The signals (Appendix Fig. S1) were quantified. After 1h of double treatment with n-butanol and ABA, the abundance of detyrosinated α-tubulin was increased. The abundance of detyrosinated α-tubulin was strongly inhibited and decreased by 97% after 2h of double treatments with n-butanol and ABA. When it was pretreated with n-butanol 1h before treatment with ABA 3h, the detyrosinated α-tubulin level was inhibited but not significant compared to the control level. However, double n-butanol and ABA treatment didn't affect the tyrosinated α-tubulin level (Fig. 3.8 C, D). ABA treatment 1h induced transiently the detyrosinated α-tubulin level, then it decreased gradually in 2h and 3h of ABA treatments. The trend of tyrosinated α-tubulin level was the same as the double n-butanol and ABA treatment (Fig. 3.8 A, B).

While the effect for detyr tubulin at 2 h looked impressive, it was just the sum of the response to n-butanol alone (seen after 2 h of addition) and the response

to ABA (seen after 1 h of addition). The interaction of ABA and n-butanol on detyrosination was additive, therefore, which meant, they went through different paths and only converged at their final point.

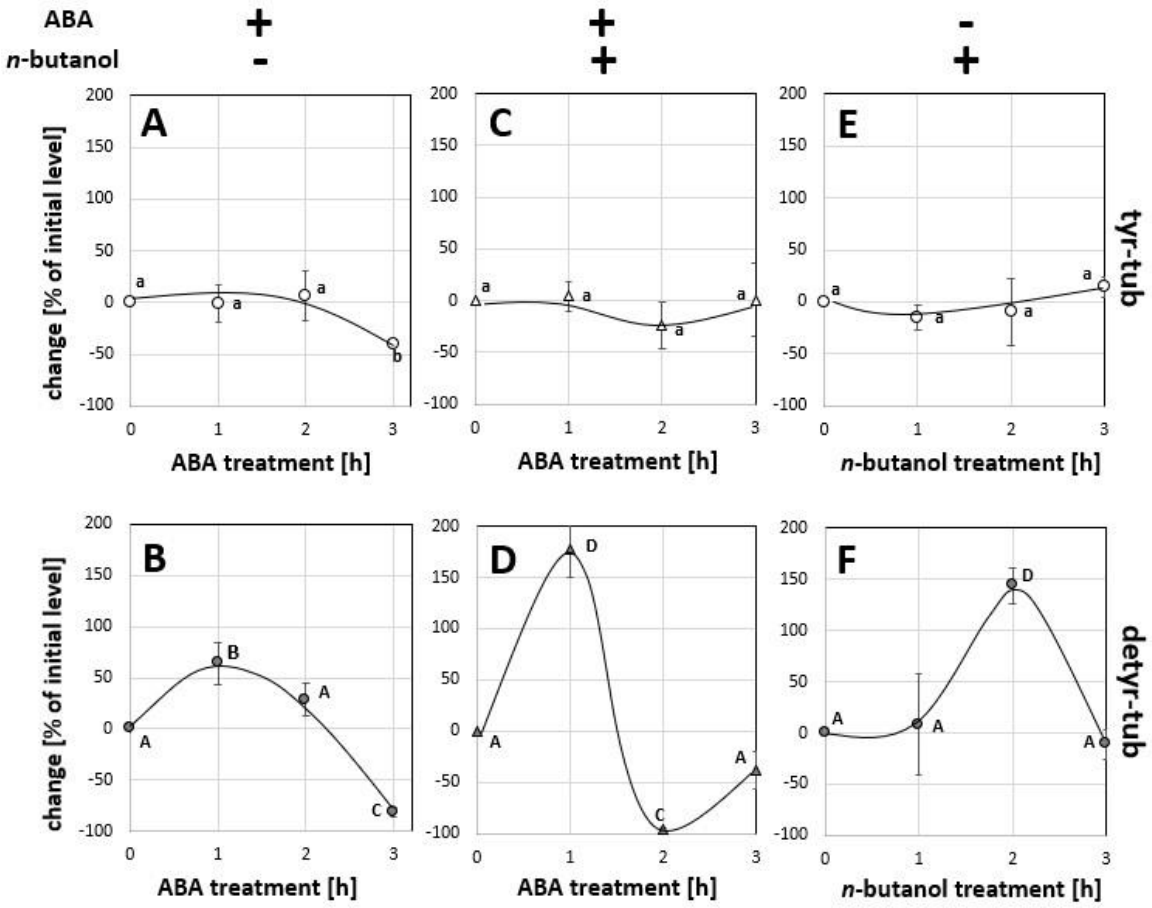


Fig. 3.8. Interaction of ABA (50 µM) and *n*-butanol (1%) concerning post-translational modification of a-tubulin in cells expressing the *NtTUA3-GFP* marker. Quantification of the relative change (in %) of the respective tubulin form as compared to the abundance seen in the control run in the adjacent lane of the blot over time of treatment. **A, C, E** tyrosinated a-tubulin, **B, D, F** detyrosinated a-tubulin. **A, B** response to ABA alone, **C, D** response to ABA after pre-treatment with *n*-butanol for 1 h, **E, F** response to *n*-butanol alone. Data represent mean and standard errors from three independent extractions. Statistical differences were tested by the Tukey LSD test, different letters indicate that the respective data point belongs to a different population at $P<0.05$.

3.1.8 The early response of detyr- tub to ABA is dependent on PLD activity

So far, we had shown that ABA not only could activate the activity of PLD enzyme but also could stimulate detyrosinated α-tubulin. Now, there was a question which part would be first activated by ABA. Next step, the Mas7, a G-protein activator, and PTX, an inhibitor of G-protein, were used to test this assumption. The tyrosinated α-tubulin and detyrosinated α-tubulin were induced after 1h of Mas7 treatment. Whereas, the regulation of tyrosinated α-tubulin was significantly stronger than that in the detyrosinated α-tubulin. In contrast, double treatment with PTX 1h and ABA 1h inhibited detyrosinated α-tubulin and tyrosinated α-tubulin levels by about 4% and 11%, respectively (Fig. 3.9; Appendix Fig. S6). Thus, from these results, we could say that ABA first stimulates detyrosinated α-tubulin.

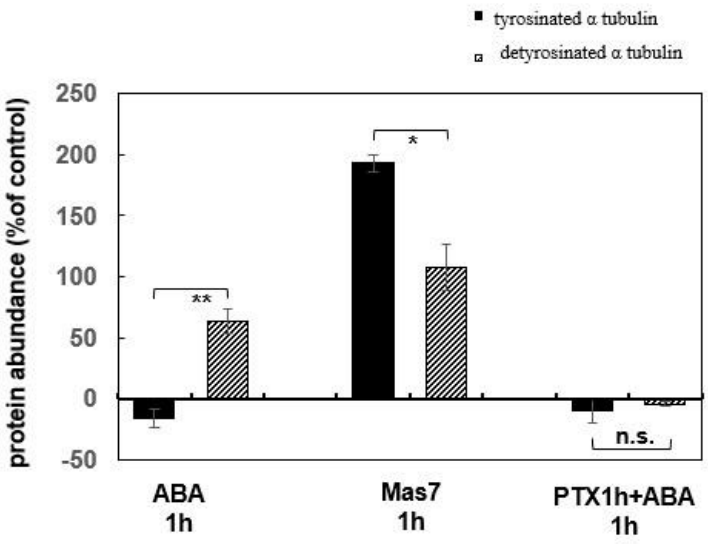


Fig. 3.9. The content of tyrosinated and detyrosinated α - tubulin was scored by plotting the % change in BY-2 cells expressing NtTUA3-GFP by antibodies ATT and DM1A after 50μM ABA for 1h, or 5μM Mas 7 for 1h, or pretreatment with 10 μg/mLPTX 1h before using ABA 1h. Data indicated mean and SE came from three biological replications and the Student's t-test for paired data was used to test the significant

difference. * indicating significant differences at P <0.05 and ** at P <0.01.

So far, all of experiments were done in the BY-2 tobacco cells. From these results, we got such a conclusion that ABA induced microtubular cold stability through modulating detyrosination/tyrosination cycle. As we all know, single cell is different from plants. So, next step, I intend to check whether ABA has effect on plants.

3.2 The function of ABA in rice growth and development

3.2.1 Genotyping and phenotypic analysis

We obtained a rice tubulin marker line overexpressing *AtTubB6-GFP* under CaMV-35S promotor (named rice TubB6 line). When we harvested its T2 generation, three independent TubB6 rice lines were selected. To ensure genetic homogeneity, the genome DNA was exacted to do the genotyping. The product size we got from genotyping was consistent with the gene length for Hygromycin 887bp and TubB6 640bp, respectively (Fig. 3.10).

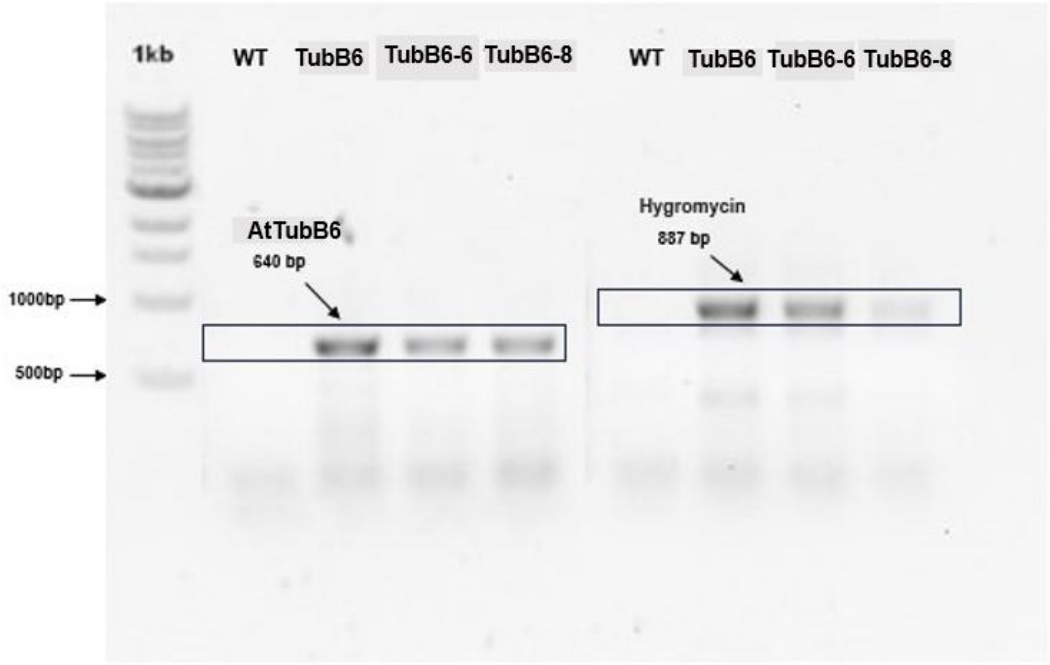


Fig. 3.10 Genotyping was done in the leaves harvested from the second generation of three independent rice transgenic tubulin marker lines named TubB6, TubB6-6 and TubB6-8 after sowing 21 days.

3.2.2 Transcript levels of *AtTubB6* in *AtTubB6-GFP* overexpressing rice plants

We selected three independent *AtTubB6-GFP* overexpression rice lines to check their transcript level by real-time RT-qPCR. From these results, line TubB6 had the highest transcript level than that in the other overexpression rice lines (Fig. 3.11). Therefore, to further identify the phenotypic readouts, we selected the line with the highest expression level to do the following experiments and is designated as TubB6 line.

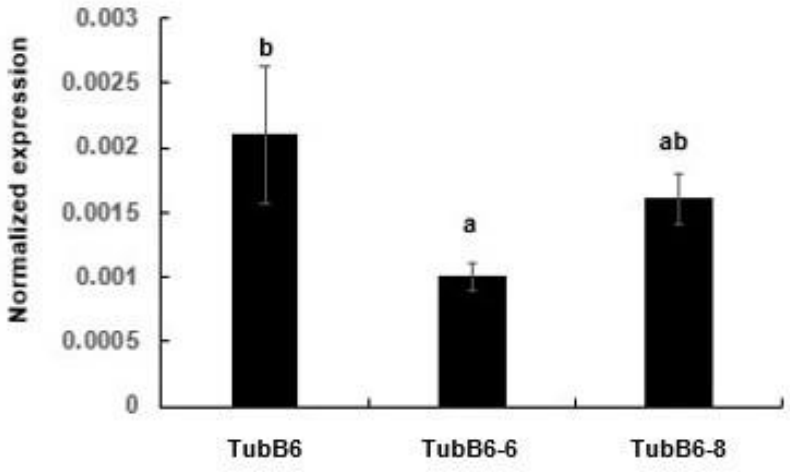


Fig. 3.11 Gene expression level of *AtTubB6* in the primary roots of three independent *AtTubB6-GFP* overexpression rice lines at day 5 after germination. Statistical differences were tested by the Tukey LSD test, different letters indicate that the respective data point belongs to a different population at $P<0.05$ Data represent mean values ± SE from three biological replicates.

3.2.3 Overexpression of *AtTubB6* inhibits rice plants growth

To understand the potential function between plants growth and tubulins, we observed the growth of roots and coleoptiles at day 5 after sowing in the wild type and TubB6 lines. The average of roots and coleoptiles was shorter than that in the WT (Fig. 3.12). We also observed the pattern in the TubB6-6 and TubB6-8 lines (Appendix Fig. S1)

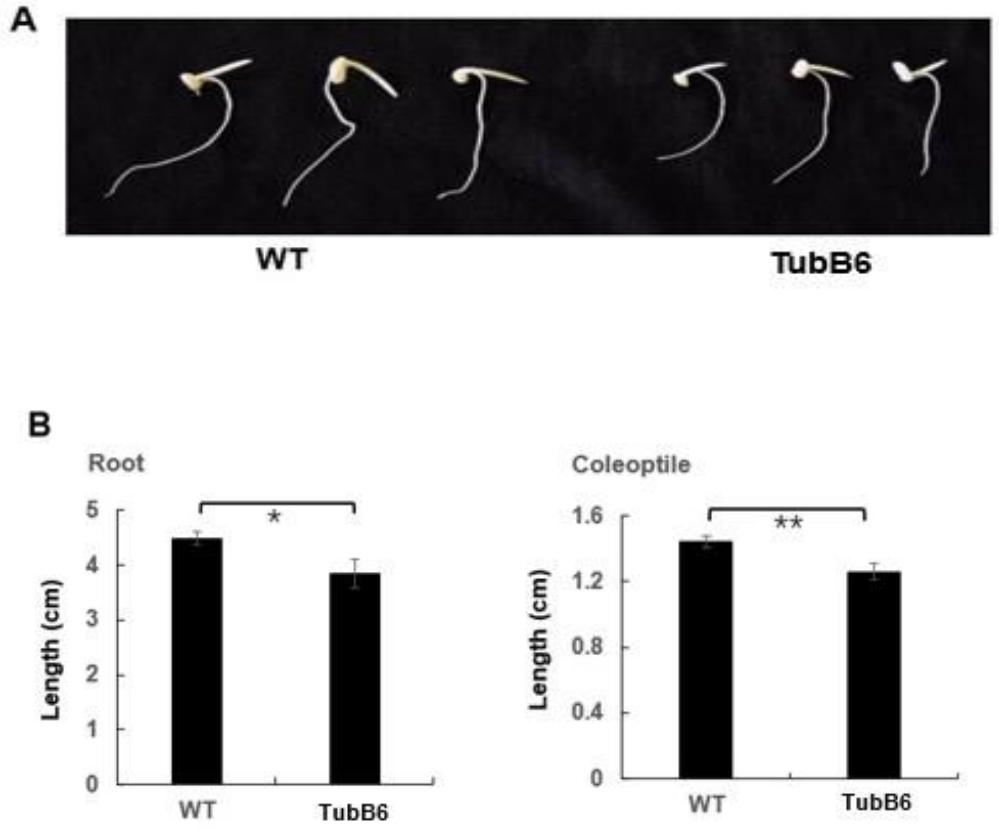


Fig. 3.12. Phenotype of *AtTubB6* rice seeding compared to WT. A. Representative image of root and coleoptile at day 5 after germination. B. Coleoptile and root length (cm) of AtTUB6 rice seedings and WT. Differences were tested for significance with a Student's t-test for paired data with * indicating significant differences at P <0.05 and ** at P <0.01. and each replicate represents seven seedlings.

Then, we observed the height of the TuB6 line and WT after 21 days of cultivation. These results demonstrated that the height of TUB6 was lower than that of the WT (Fig. 3.13 A, C). We also detected the leaf inclination between WT and TuB6 lines, resulting in a smaller leaf inclination in the TuB6 line, when compared to WT (Fig. 3.13 A, B).

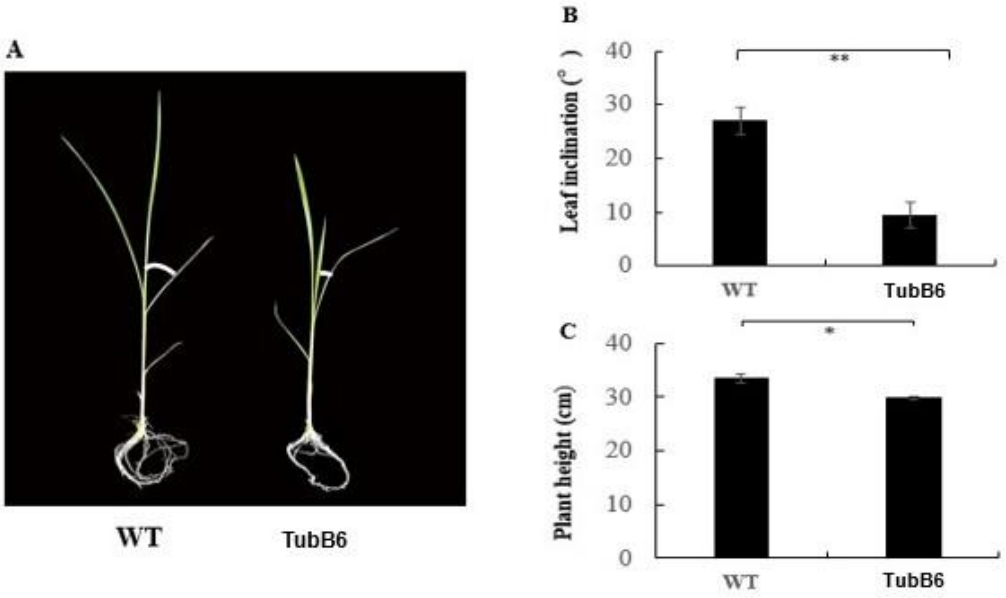


Fig. 3.13 Phenotypic analysis in the rice plants expressing *AtTubB6-GFP.* Observation of the 21-day-old plants of the wild type and TubB6 line under normal conditions, respectively (**A**); Quantification of leaf inclination (**B**) and plant height (**C**). Data represent mean and SE from three biological replications. Differences were tested for significance with a Student's t-test for paired data with * indicating significant differences at P <0.05 and ** at P <0.01.

3.2.4 The overexpression of *AtTUB6-GFP* regulates plant growth via modulating detyrosinated/tyrosinated α-tubulin

Microtubules are composed of α-tubulin and β-tubulin, which are the target of the post-translational modifications (PTMs). The detyrosination/tyrosination cycle is the most common post-translational modification, which can control cell division and cell elongation. Therefore, we want to test whether the overexpression of *AtTubB6* leads to the change of detyrosinated/tyrosinated α-tubulin level to affect plant growth. We performed a western blot and used primary antibodies ATT and DM1A to prob detyrosinated and tyrosinated α-tubulin levels. The signals were quantified. In coleoptiles, the abundance of tyrosinated α-tubulin induced by around 30% and the detyrosinated α-tubulin

level decreased by 27%. In roots, both the abundance of tyrosinated α-tubulin and detyrosinated α-tubulin decreased by around 80% and 24%, respectively (Fig. 3.14).

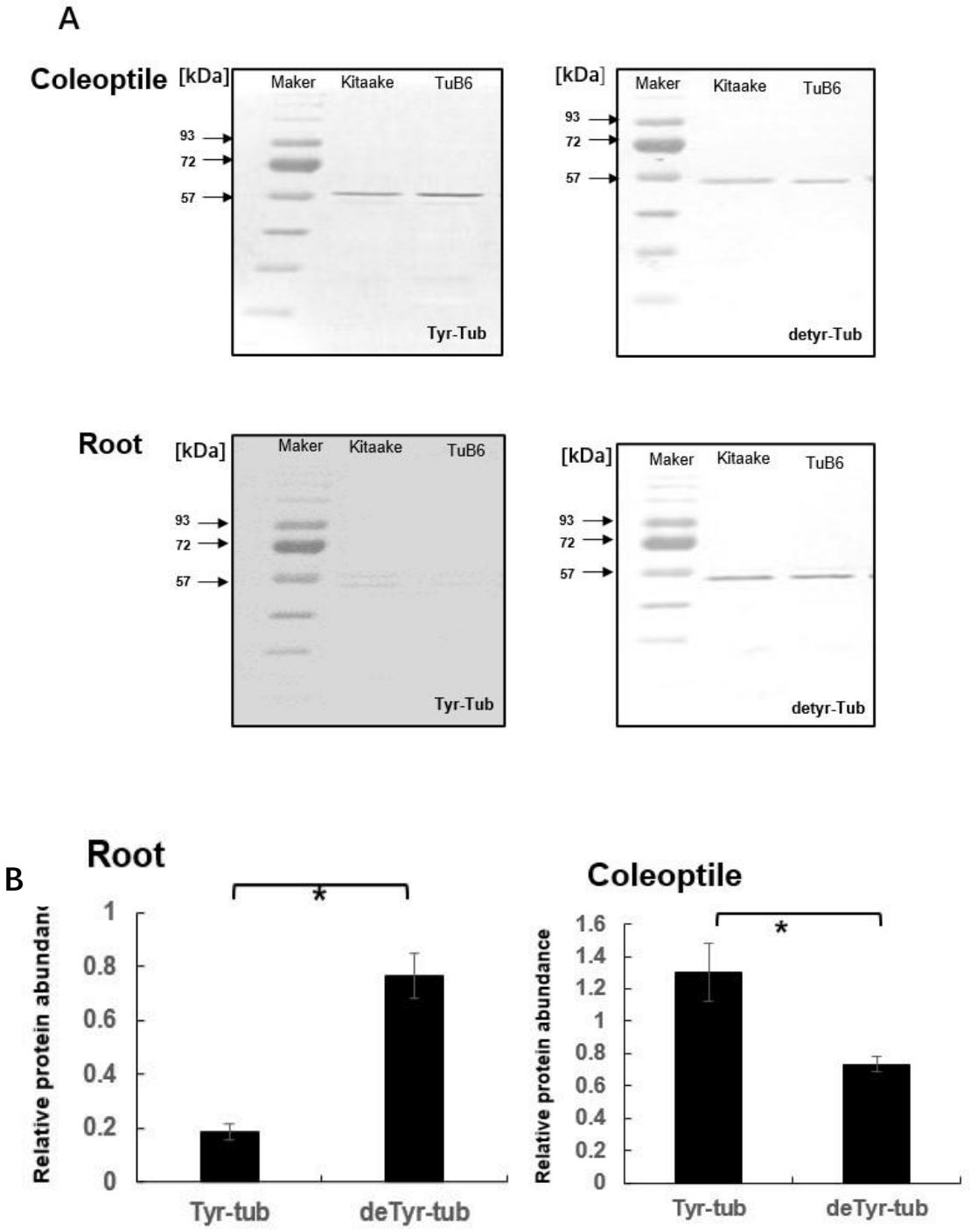


Fig. 3.14 Detection of western blot in total extracts from coleoptiles and seminal roots of kitaake (WT) and the TubB6 line at day 5 after germination. A. Representative Western

blots after probing with monoclonal antibodies against tyrosinated a-tubulin (tyr-tub) and detyrosinated a-tubulin (detyr-tub) for kitaake (WT) and the TubB6 rice seedings. B. Quantification of tyrosinated and detyrosinated α‐tubulin content from coleoptiles and seminal roots of TubB6 overexpressor relative to Kitaake (defined as 1). Data represent mean and SE from three biological replications. Differences were tested for significance with a Student's t-test for paired data with * indicating significant differences at P <0.05 and ** at P <0.01.

3.2.5 ABA interacts with AtTubB6 to accelerate the inhibition of Plant Growth

To get insight into the role of ABA in plant growth, we observed the growth of roots and coleoptiles of the TubB6 line and wildtype on day 5 after sowing in the ABA condition or without ABA. Here, we observed that both of the growth of roots and coleoptile was inhibited in TubB6 rice lines and the wildtype under ABA treatment. However, the degree of inhibition of roots and coleoptiles in TubB6 line was higher than that in wildtype in presence of ABA (Fig. 3.15). We also observed the same pattern in the TubB6-6 and TubB6-8 lines compared to wildtype under ABA condition (Appendix Fig. S2)

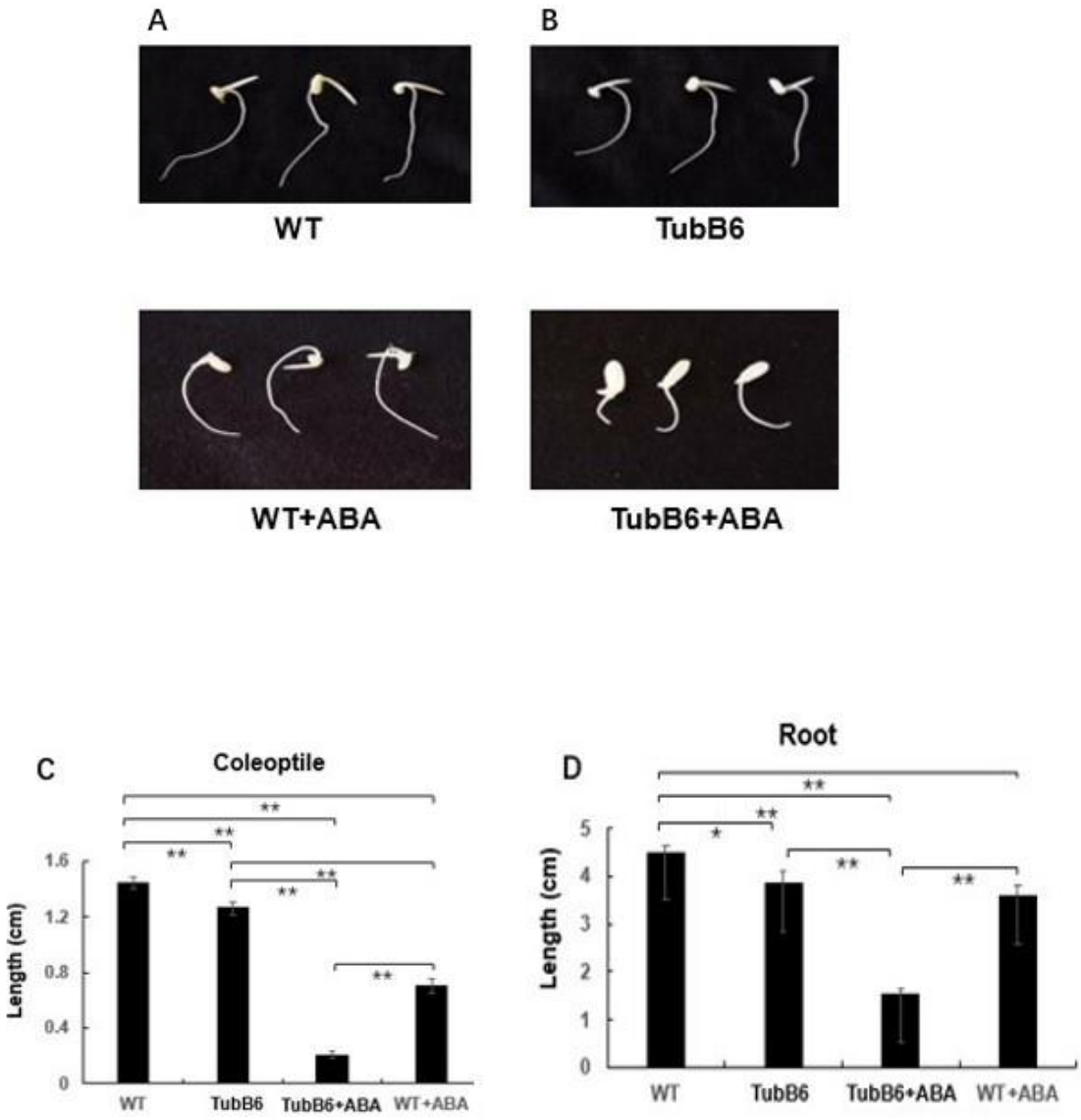
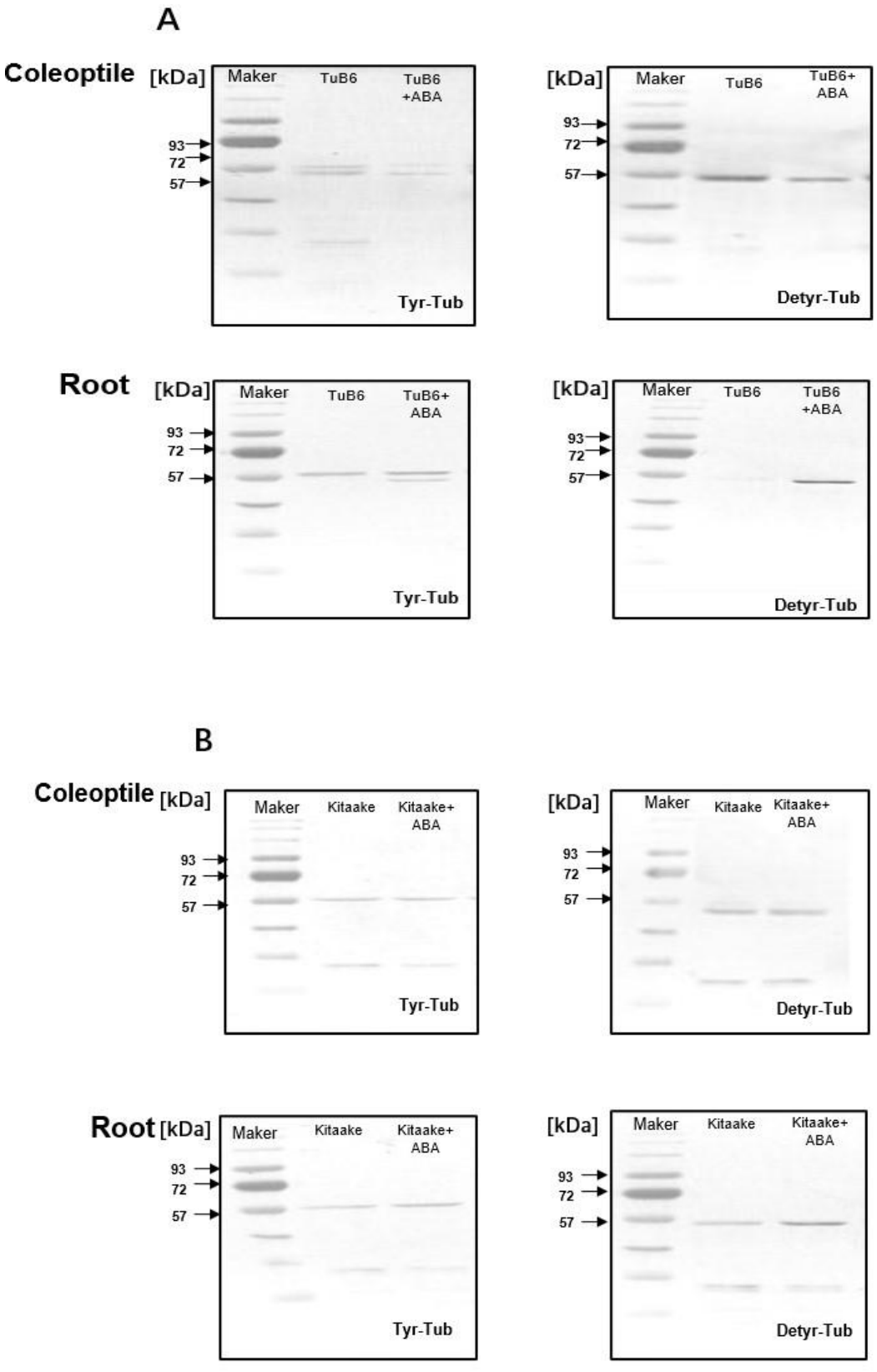


Fig. 3.15 Phenotype of TubB6 rice seeding compared to WT. Representative image of root and coleoptile of TubB6 line (A) or wild type (B) at day 5 after germination either in the absence or presence of ABA. Coleoptile (C) and root (D) length of TubB6 rice seedings and WT either in the absence or presence of ABA. Differences were tested for significance with a Student's t-test for paired data with * indicating significant differences at P <0.05 and ** at P <0.01.

3.2.6 ABA accelerates the inhibition of Plant Growth through regulating detyrosinated α-tubulin.

Before we have observed that the overexpression of *AtTubB6* can inhibit the growth of roots and coleoptiles by modulating the detyrosination/tyrosination cycle. Moreover, ABA can interact with *AtTubB6* affects the inhibition of plant growth. Therefore, next step, we would like to test whether higher inhibition of plant growth by ABA interaction with *AtTubB6* lead to the change of detyrosinated/tyrosinated α-tubulin level. We did this experiment in the rice TubB6 line as well as wildtype. The total proteins were extracted from coleoptiles and roots of TubB6 line and wild type at day 5 after sowing with ABA treatment. Through western blot, we used two monoclonal antibodies, ATT and DM1A, to detect tyrosinated α-tubulin and detyrosinated α-tubulin, respectively. We quantified the band signals and compared them on a relative scale. In the coleoptiles of TubB6 line, in the presence of ABA, the relative content of detyrosinated α-tubulin decreased by 60%, however, the tyrosinated α-tubulin level didn’t change upon the TubB6 line without ABA treatment. In the roots of TubB6 line, the detyrosinated α-tubulin was induced by 110% in ABA treatment compared with the TubB6 line without ABA treatment, while there was no different in the tyrosinated α-tubulin content (Fig. 3.16 C). In wildtype, no matter whether in the coleoptile or in the roots, we found that both of detyrosinated α-tubulin level and tyrosinated α-tubulin level didn’t change (Fig. 3.16 D).


A
Coleoptile
[kDa]
Maker
TuB6
TuB6 +ABA
93
72
57
Tyr-Tub
[kDa]
Maker
TuB6
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Detyr-Tub
Root
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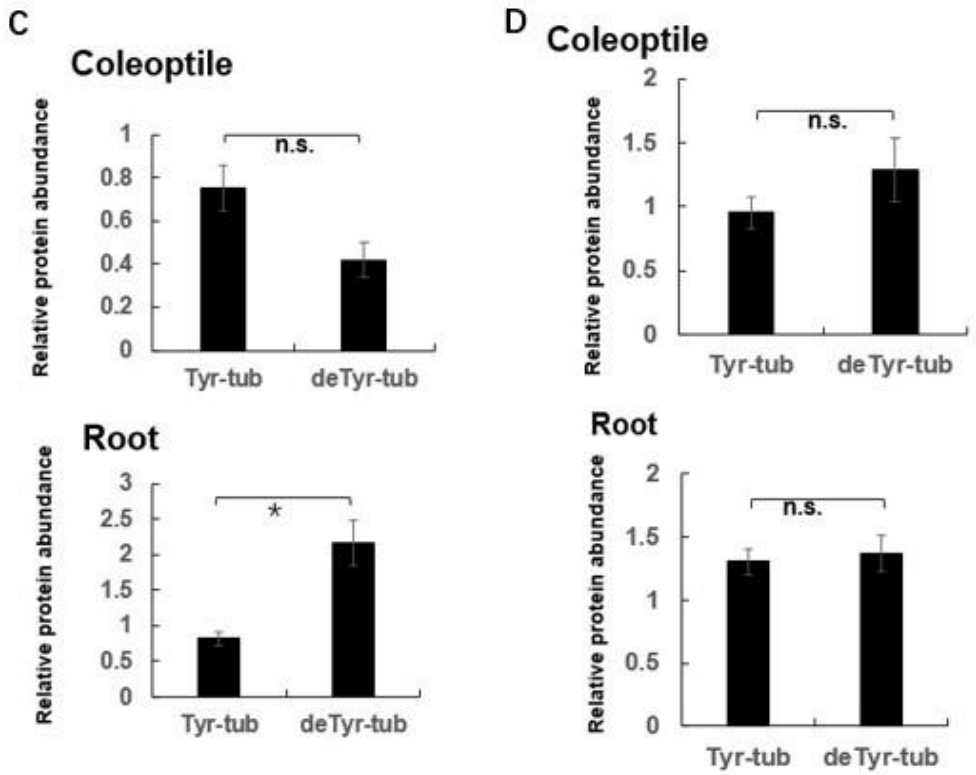

Fig. 3.16 Detection of tyrosinated and detyrosinated α-tubulin by antibodies ATT and DM1A in total extracts from coleoptiles and seminal roots of rice TubB6 line (A, C) and wildtype (B, D) in presence of 1μM ABA at day 5 after germination. Quantification of tyrosinated and detyrosinated α‐tubulin content from coleoptiles and seminal roots of TubB6 line with ABA treatment relative to the TubB6 line without ABA or wildtype with ABA treatment relative to the TubB6 line without ABA or wildtype without ABA treatment (defined as 1). Data represent mean values ± SE of three replicates from coleoptile and root. Differences were tested for significance with a Student's t-test for paired data with * indicating significant differences at P <0.05 and ** at P <0.01 and ns non-significance at P >0.05

4. Discussion

In the present study, ABA resulted in changes in plant growth and development as well as stress tolerance through affecting the distribution of tubulin detyrosination/tyrosination. Therefore, in the following sections, we discussed the role of ABA in the rice plants and BY-2 cell lines, respectively.

First, in rice plants, as most specific phenotype, the application of ABA inhibited growth of roots and coleoptiles of rice plants overexpressing AtTUB6-GFP. Subsequently, it discussed the significant findings due to the application of ABA with respect to the change of detyrosinated/tyrosinated α-tubulin which can lead to inhibition of plant growth.

Second, we focused the role of ABA on tobacco BY-2 cell lines. ABA increased the resistance of microtubules under cold stress. Next, the function of ABA in response to microtubules under cold stress through post-translational modification was discussed. After that, the triangular relationship in the ABA, PLD and detyrosinated α-tubulin, and the interesting findings about a rice tubulin tyrosine ligase-like 12 protein (TTLL12) that participated in this loop were also discussed. Finally, we discussed whether OsTTL has the function of tyrosine ligase and also stated how we need to confirm this hypothesis in future studies.

4.1 A role of ABA on plant growth

Plant hormone, ABA, is usually thought to have the function to control the various growth and developmental processes of plants, for example, cell division and cell differentiation, as well as it is also proposed as a mediator which can induce plant response to stressful environments (Jia *et al*., 2011;

Wang *et al*., 2011; Ye *et al*., 2012). For the first aspect, there are two lines of evidence to support the role of ABA in the regulation of plant growth. First, steeply oblique microtubule bundles are observed when Chinese winter wheat (Triticum aestivum L.) is exposed to 50μM ABA condition (Wang and Nick, 2001). Second, ABA affects the closure of stomata through disrupting cortical microtubules (Jiang *et al*., 1996). In parallel, the detyrosinaton/tyrosination cycle acts as the most common post-translational modification, which can recruit a set of specific microtubules-associated proteins (MAPs) resulting in the effects on the structural and dynamic properties of microtubules controlling the cell division and cell elongation (Nick, 2007; Peris *et al*., 2009; Cai, 2010; Zhang *et al*., 2021). This leads to a question, what is the relationship between ABA and the detyrosination/tyrosination cycle?

In order to investigate this hypothesis, we used ABA in the current study, to treat rice plants overexpressing TUB6-GFP. The application of ABA not only inhibited root growth but also prevented the growth of coleoptiles (Fig. 3.15). Then, we also measured the detyrosinated/tyrosinated α-tubulin level in this condition. Interestingly, the effect of ABA on the distribution of detyrosinated/tyrosinated α-tubulin was different between roots and coleoptiles. In roots, the detyrosinated α-tubulin was favoured. In coleoptiles, both detyrosinated and tyrosinated α-tubulin decreased, but the level of tyrosinated α-tubulin was higher compared to detyrosinated α-tubulin (Fig. 3.16 A, C). Our results were consistent with the funding by Jovanovic *et al*., (2010), where the mitotic indices of roots in rice plants would be decreased significantly leading to induce more tyrosination of tubulin. Now, there is a contradiction. Because in our data, the detyrosinated α-tubulin was induced by ABA, whereas, the tyrosinated α-tubulin decreased. For this situation, two reasons can explain this contradiction. First, in the root, detyrosinated α-tubulin is favoured. Second, detyrosination can not only a factor to regulate plant growth, it should interact with other microtubule-

associated proteins (Hamada, 2014; Zhang *et al*., 2021). In coleoptile, the decrease of detyrosinated α-tubulin was stronger than that of tyrosinated α-tubulin (Fig. 3.16 A), we could conclude that coleoptile correlated with a higher proportion of tyrosinated α-tubulin, which was consistent with the result from Wiester *et al*., 2002. There was a study that demonstrated that longitudinal microtubules were linked to detyrosination which could be induced by auxin depletion. Maybe this finding was relevant to this fact, where we incubated the rice plants overexpressing TUB6-GFP for about 21 days under normal conditions, causing the decrease of leaf inclination and inhibition of plant height (Fig. 3.13).

4.2 A role of ABA on cold stability of microtubules

For the second aspect, ABA has an important role in the adaptation of plants to cold stress. The induction of cold resistance of microtubules will result in the accumulation of ABA content (Wang & Nick, 2001). Now, the question is how does the ABA accelerate the resistance of microtubules under cold stress. To get insight into the function of ABA in the cold resistance of microtubules, we used ABA, in the present study, to treat overexpressed tubulin marker gene TUA3 in the tobacco BY-2 cells in the absence or presence of cold stress. We observed that ABA treatment for 60 min inhibited the disassembly of cortical microtubules under cold conditions (Fig. 3.1 A, B, D, E), which was matched with a report that the pretreatment with ABA enhanced the cold resistance of microtubules (Lee & Chen, 1993; Sakiyama & Shibaoka, 1990; Tanino *et al*. 1990). In contrast, The ABA inhibitor, AA1, accentuated the cold-induced elimination of microtubules (Fig. 3.1 A, C, D, F). This is consistent with the fact that ABA focus on increasing microtubules stable against cold stress (Wang *et al*., 2011). The ability of ABA to induce microtubular cold stability may be due to the increase of ABA level induced by cold stress, which can result in the

induction of specific proteins that are responsible for the increased cold stability (Chen *et al*., 1983; Robertson *et al*., 1987)

For most plant species, cold adaptation is a slow process which usually takes 3-6 weeks to finish the whole development (Chen *et al*., 1982; Gusta *et al*., 1982). However, pretreatment with ABA accelerates the cold tolerance of Norstar winter wheat cells and it only requires 4 days to adapt to -30'C conditions (Chen & Gusta, 1983). Furthermore, the decrease of protein and MT levels induced by ABA also occurs in the initial stage of plant cold acclimation (3℃, 2-3 days) (Olinevich & Khokhlova, 2003). Evidences were given to support this point in Figure 3.3, when we extended the pretreatment time of ABA to 120 minutes and 180 min before putting the TUA3 cell line into a cold environment, the function of stabilizing the microtubules of ABA disappeared under cold stress. maybe it is because signals trigger the processes to adapt low-temperature. With the extension of cold acclimation time, the role of ABA in the formation of plant cold tolerance decreased. In this situation, other hormone-independent mechanisms of cold-resistant development are triggered, including the role of cytoskeleton components and cytoskeleton related protein (Olinevich & Khokhlova, 2003).

4.3 ABA interacts with PLD to affect the cold stability of microtubules

In our previous work, we had shown that the PLD binded to detyrosinated α-tubulin (Zhang *et al*., 2022). PLD, a 90 kDa protein which is isolated from tobacco BY-2 membranes, not only can decorate microtubules in plant cells but also has been demonstrated to act as a signal hub to link with the microtubular response to cold stress (Munnik *et al*., 2003: Gardiner *et al*., 2001). Before, we

have shown that ABA had the ability to induce cold stability of microtubules. So, it gave rise to a question: how did the function of PLD in the cold response of microtubules under ABA treatment? It is still not clear now. We, therefore, probed for the effect of PLD by detecting the enzymatic activity of PLD under ABA conditions and cold stress conditions, respectively. We found that ABA 60 minutes and cold 60 minutes increased PLD activity significantly over the resting level (Fig. 3.4 A). Interestingly, we also found that by extending the treatment time to 180 min, the PLD activity had already fully dissipated (Fig. 3.5). So, this effect was transient. This is linked to the fact that PLD acts as an early step of cold signaling to increase plant tolerance (Zhang *et al*., 2022). It has been demonstrated that phospholipase activity of PLD is dependent on the rapid increase of intracellular level, which can be regulated by cold stress (Ruelland *et al*., 2002). Furthermore, the PA a second messenger, is produced by PLD which can hydrolyze structural phospholipids, which can mediate ABA-induced ion channels to close stomata in plants, resulting in insensitivity of cells of microtubules to ABA treatment (Jacob *et al*., 1999: Xing *et al*., 2009).

4.4 ABA induces detyrosinated α-tubulin is dependent on PLD activity

So far, we had identified ABA might correlated with PLD activity to regulate microtubular cold stability. Krtková *et al*., 2012 discovered a specific PLDα1 from ethyl-N-phenylcarbamate (EPC) affinity chromatography by co-elution with detyrosine-tubulin in the presence of high ionic stringency, indicating that it specifically binds to the modified form of tubulin. Interestingly, this study also found that PLDα1 didn’t bind to tyrosine tubulin, although in the much lower ionic stringency. Then, Zhang *et al*., 2022 proved that OsPLDα1 might be associated with detyrosinated α-tubulin in BY-2 cell lines overexpressing OsPLD-GFP. Our previous work had shown that the activity of the PLD enzyme

could be activated transiently by ABA application. Now, there is a question: whether there is a relationship between ABA and detyrosinated α-tubulin. To test this relationship, we used monoclonal antibodies ATT and DM1A to detect the tyrosination of a-tubulin and detyrosination of α-tubulin, respectively. We found that ABA 1h first increased significantly the detyrosination of α-tubulin level, and when prolonged the treatment time to 3h, the effect was almost disappeared (Fig. 3.6). So, the effect of ABA on the detyrosination of α-tubulin was also transient. In last step, we have discussed that ABA could activate activity of PLD enzyme. And the OsPLDα1 overexpressed in tobacco cells showed an increase of detyrosinated α-tubulin (Zhang *et al*., 2022). We, therefore, proposed that the application of ABA not only could stimulate the activity of PLD enzyme but also could induce detyrosination of α-tubulin level. ABA had a transient effect on PLD activity that in turn activated detyrosinated α-tubulin, so the effect of ABA on detyrosinated α-tubulin was also transiently. According to the functional link in the ABA, PLD and detyrosinated α-tubulin, we gave rise to another question: if was the increase of detyrosinated α-tubulin level due to the activation of PLD enzyme activity by the ABA treatment? To further test this possibility, the activator or inhabitation of PLD enzyme would be used in the following vivo studies.

The activation of PLD requires a trimeric G protein (Wang. 2005), which in turn activates the intracellular effector enzymes, leading to the formation of second messenger (Birnbaumer *et al*., 1990; Berridge, 1993; Nishizuka,1995). The activation or inhibition of G-protein can be done by mastoparan and pertussis toxin, respectively (Dhonukshe *et al*., 2003). To test the hypothesis before we proposed, we treated with 10 μg/mL pertussis toxin before putting the TUA3 cell line into ABA 1h, showing there was no change in the detyrosination α-tubulin level. Whereas in the double treatments with 5 μM Mas 7 and ABA,

detyrosination α-tubulin level was induced significantly (Fig. 3.9). Based on these results, we proposed that ABA stabilizes microtubules through a PLD-dependent detyrosination pathway.

In summary, ABA not only has the function to stabilize microtubules under cold stress but also can affect the detyrosination/tyrosination cycle through correlating with PLD activity although these effects are transient.

4.5 what are the plant TTLs?

Apart from the above results, another important signal hub is a rice tubulin tyrosine ligase-like 12 protein (TTLL12) (Zhang *et al*., 2021). Now, there is a question: what is the TTLL12 protein in plants and why is it worth studying?

In plants, cell division, cell growth as well as cell differentiation are dependent on the dynamic reorganization of microtubules (Nick, 2007). This raises the question of how to allocate tubulins to different microtubule arrays that coexist partially in the same cell. Based on the discovery that microtubules use different isotypes at the same time, the original idea that different arrays are composed of different tubulin isotypes has been abandoned (Hussey *et al*., 1987). However, post-translational modification of tubulin will provide another mechanism for assigning different functions to different microtubules in the same cell. The highly conserved tyrosine at the carboxyl terminal of all eukaryotic tubulins (Watts *et al*. 1988) proves the importance of microtubule tyrosine and tyrosine.

The detyrosination/tyrosination cycle is the most common PTM (Zhang *et al*., 2021). In all eukaryotes, the shift of detyrosinated α-tubulin to tyrosinated α-tubulin is because the conserved C-terminal tyrosine of α-tubulin is removed by

an elusive tubulin tyrosine carboxypeptidase (TTC), in turn, when there exist the enzyme, tubulin tyrosine ligase (TTL), tyrosinated α-tubulin will be produced (Ersfeld *et al*., 1993). Because of the lack of the homologs of vasohibins (VASH), which is the regulator responsible for the detyrosination/tyrosination cycle in plants, the study of plant TTC remains a molecular enigma (Zhang et la., 2021). However, it found that there seem to be molecular homologs of TTL in plants. In animals, besides bona fide TTL protein, one of the numbers of TTL protein families, which is identified to have the function to re-tyrosinate α-tubulin, the others have the function of glutamylases including TTLL1, 2, 4, 5, 6, 7, 9, 11, and 13, or glycylases containing TTLL3, 8, and 10 (Janke and Magiera, 2020; Zhang *et al*., 2021). Among them, human TTLL12 is thought to be an atypical TTL family. Because it just affects detyrosination levels and does not affect glutamylase and glycylase (Wasylyk *et al*., 2010; Brants *et al*., 2012). It is reported that in eukaryotes, the TTLL12 family is conserved and seems to be the only TTL family in plants (Janke *et al*., 2005).

4.6 role of OsTTL in cold stability of microtubules induced by ABA

To test the function of TTLL12 in plants, in our lab, a rice plant overexpressing OsTTLL12-RFP was constructed and the results showed that OsTTLL12 binds to detyrosinated α-tubulin (Zhang *et al*., 2021). Now, based on this conclusion, it appears a new question: does ABA affect the detyrosination/tyrosination cycle through TTLL12 protein? To address this question, we used a suspension culture of tobacco (Nicotiana tabacum L. cv Bright Yellow-2, BY-2) cell co-expressing *NtTUA3-GFP/OsTTLL12-RFP* to test this hypothesis. ABA treatment didn't alter the detyrosination/tyrosination of α-tubulin. These results were completely different from that in the ABA treatment of the *NtTUA3-GFP* cell line (Fig. 3.7). For this situation, we shouldn't forget that α-tubulin is already

in the co-expressing *NtTUA3-GFP/OsTTLL12-RFP* cell line. The TTLL12 protein is linked to the detyrosinated α-tubulin and also interferes with PLD (Krtková *et al*., 2012), which would explain why the application of ABA affects the cycle of detyrosination/tyrosination of α-tubulin which is dependent of TTLL12 protein. In brief, when ABA is used to the co-expressing *NtTUA3-GFP/OsTTLL12-RFP* cell line, according to our above results, it will induce a cascade reaction, where ABA will stimulate PLD activity resulting in increasing detyrosination α-tubulin level. At the same as TTLL12 protein is already in there also can induce PLD activity and detyrosination α-tubulin level. We, therefore, think ABA should regulate TTLL12 protein.

4.7 the relationship between rice TTL and TTC

N-butanol is a primary alcohol and has the property of activating PLD activity, which can produce phosphatidic acid (PA). Apart from the production of PA by n-butanol treatment, it also can consume PA. In brief, when n-butanol activates PLD as the same as n-butanol itself also can act as a trans-phosphatidylation substrate (Munnik *et al*., 1995; Dhonukshe *et al*., 2003). Because of the specific function of n-butanol which can consume PA, and connect the fact, where the PA also can recruit MAP65-1. Therefore, when PA was consumed by n-butanol, it can result in lower MAP65-1 which will compete with C-terminal binding sites of α-tubulin with OsTTLL12. leading to the increase of OsTTLL12 level. Since more OsTTLL12 will prevent the inhibition of tyrosinated αβ tubulin dimmers on the translation of α tubulin, such that the detyrosinated α-tubulin will be induced (Fig. 4.1) (Zhang *et al*., 2022). In our work, 55kDa size protein detected by western blot using antibodies ATT and DM1A respectively proved that the content of detyrosinated α-tubulin was induced significantly by n-butanol treatment alone at 2h (Fig. 3.8 E, F). The result we got was consistence to the fact, where n-butanol could activate PLD activity that binds to detyrosinated α-

tubulin (Zhang *et al*., 2022). Therefore, we proposed that in the present of n-butanol, detyrosinated α-tubulin was favored such that producing more TTC not TTL. There is a conditraction with the evidence that OsTTLL12 as the most likely plant homologue for a TTL function can lead to higher level of detyrosinated α-tubulin (Zhang *et al*., 2021). We guessed whether TTL is a TTC. If this assumption is correct, the results we got seem to be reasonable.

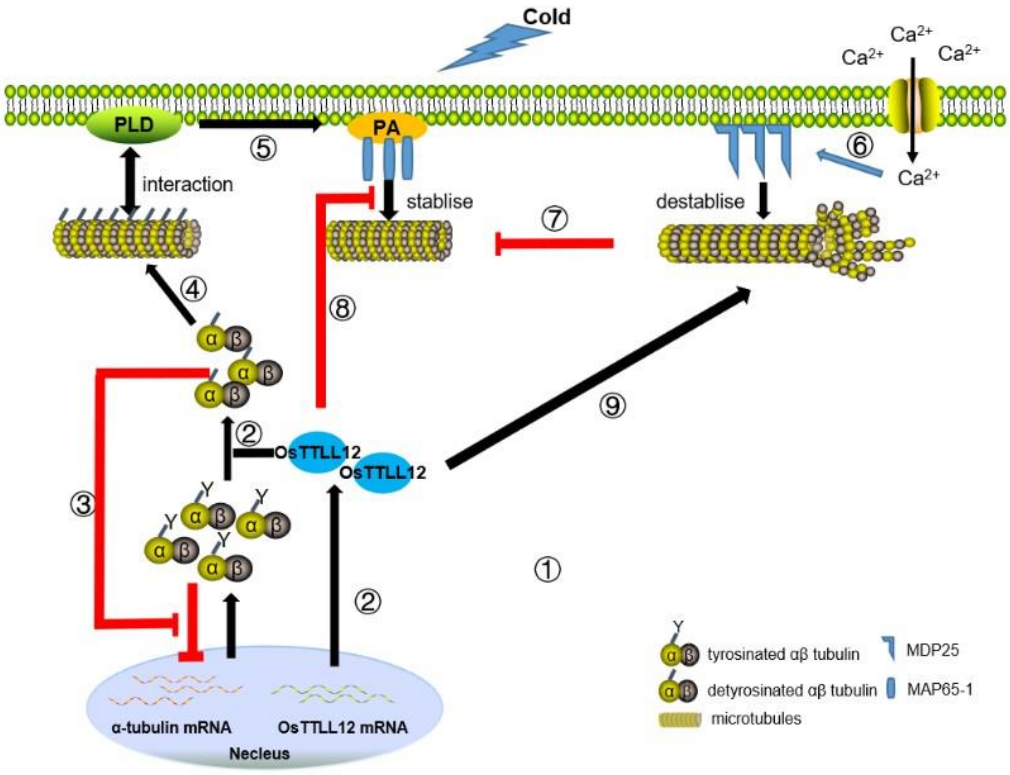

Fig. 4.1 A model of tubulin detyrosination regulated by cold signal and tubulin synthesis.

C-terminal tyrosine can be encoded by α-tubulin, and tyrosinated αβ-tubulin dimers can in turn act as inhibitors, preventing the translation of α-tubulin mRNA into protein(①). Due to the OsTTLL12 overexpressing, the OsTTLL12 protein increases to result in more detyrosinated αβ-tubulin dimers (②) which can inhibit the prevention of tyrosinated αβ-tubulin dimers on the translation of α-tubulin (③). As the same as detyrosinated αβ-tubulin interacts with phospholipase D (PLD) (④). In the presence of cold stress, PLD is activated and produces phosphatidic acid (PA) (⑤) leading to the stabilization of microtubules

through recruiting MAP65-1 protein. In parallel, cold stress can stimulate Ca^{2+} influx to activate MDP25 protein to destabilize microtubules (⑥) resulting in decreasing MAP65-1 function (⑦). C-terminal binding sites of α-tubulin are competed by OsTTLL12 and MAP65-1 proteins, therefore, when OsTTLL12 is overexpressed, the couples of MAP65-1 and microtubules will be prevented, thus promoting the destabilizing function of MDP25 on microtubules (⑨).

Although TTL has been found in the porcine brain, the TTC is still unclear (Ersfeld *et al*., 1993). The vasohibins (VASHs) as well as their regulator the Small Vasohibin Binding Proteins (SVBPs) have been shown to act as the long-time elusive tubulin carboxypeptidases (TCPs) (in plant models also abbreviated as TTC) . But, so far, there are no homologues of these animal TCPs in plants (Aillaud *et al*., 2017; Nieuwenhuis *et al*., 2017). From the model in Fig. 4.2, the enzyme TTL prefers tyrosinated tubulin and enzyme TTC can yield detyrosinated tubulin (Zhang *et al*., 2021). If we assume that TTC is present in plants. When there exists more TTL, more tyrosinated tubulin will be produced. Whereas, the overexpression of TTC will lead to more tyrosinated tubulin. Combined with our data, in presence of ABA, the overexpression of *OsTTLL12* decreased detyrosinated tubulin at 3h, whereas, the tyrosinated tubulin increased at 3h (Fig. 3.7 C, D). So, we proposed that OsTTL might be also the TTC. Moreover, a TTC inhibitor named parthenolide has been found to decrease tubilin detyrosination contents in BY-2 tobacco cells (Schneider *et al*., 2015) further suggesting TTL is a TTC.

However, we should keep in mind that the post-translational modification and tubulin protein synthesis may be involved to increase tubilin detyrosination contents caused by OsTTL (Breviario and Nick, 2000). It has shown that the complexes will be formed due to the combination between LRR domain characteristic of OsTTLL12 (Zhang *et al*., 2021) and other proteins. because

this LRR domain is different from the set-like domain of animals. It is conceivable that this interaction may guide the direction of the enzymatic reaction (connecting tyrosine vs. cutting tyrosine). In order to obtain the biochemical pathway of enzyme activity given by TTLL12 and potential binding partners, the recombinant expression will be carried out in future in vitro research.

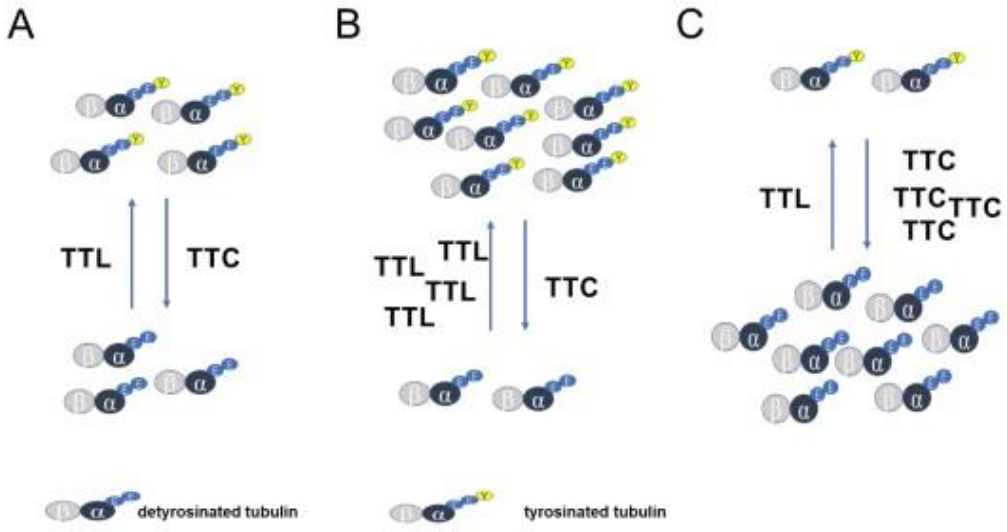

Fig. 4.2 The model for the distribution of detyrosination and tyrosination by enzyme TTL or TTC. A. Both detyrosinated α-tubulin and tyrosinated α-tubulin exist in the natural condition. But normally, there is a slightly higher amount of tyrosinated α-tubulin. B. More enzyme TTL results in more tyrosinated α-tubulin. C. More enzyme TTC leads to more tyrosinated α-tubulin, if TTC is present.

4.8 the effect of ABA on detyrosinated α-tubulin is independent on the n-butanol pathway

So far, we have addressed this fact, where ABA would deploy activation of G-protein, resulting in triggering PLD activation, which can interact with TTLL12

protein to regulate the detyrosination/tyrosination cycle. However, the phosphatidic acid (PA), a production of PLD activation, can couple with MAP65-1 protein that can compete for C-terminal binding sites of α-tubulin with TTLL12 protein to affect detyrosinated α-tubulin level (Zhang *et al*., 2022). To investigate whether the role of ABA and n-butanol have the same response path to detyrosinated α-tubulin, in our study, 55 kDa protein detected by western blot using antibodies ATT and DM1A respectively proved that the effect of ABA and n-butanol on detyrosination is additive. After 2h, the effect of detyrosination is just the sum of the response to n-butanol alone (seen after 2h of addition) and the response to ABA (seen after 1h of addition) (Fig. 3.8), suggesting that the role of ABA in response to detyrosination is independent on the n-butanol pathway.

5. Conclusion

Abscisic acid, a plant hormone, has two functions: (1) can induce cold stability of microtubules (Wang and Nick, 2001) and (2) inhibit plant growth (Brookbank *et al*., 2021). Therefore, we want to detect how ABA play these two roles.

For the first situation, ABA application can suppress the depolymerization of microtubules under cold stress, and the dynamic properties of microtubules are regulated by the detyrosination/tyrosination cycle. Through analysis of overexpression of TUA3-GFP in tobacco BY-2 cell line in the presence or absence of ABA under cold conditions, unexpectedly, ABA transiently induced cold stability of microtubules. N-butanol treatment alone as well as the duel treatments with n-butanol and ABA in a tubulin marker line *NtTUA3-GFP* BY-2 cells have shown that the effect of ABA on detyrosinated α-tubulin was independent of n-butanol pathway. Apart from these conclusions, the application of ABA in a double transgenic BY-2 cell line overexpressing *NtTUA3-GFP/OsTTLL12-RFP* allowed the detection of the role of OsTTLL12, where it was necessary for the activation of detyrosinated α-tubulin by ABA application. The distribution between detyrosinated α-tubulin and tyrosinated α-tubulin is dependent on the cycle of enzyme TTL and enzyme TTC.

Second, in previous results of our lab, it had shown that plant growth was controlled by the post-translational modification. one of the post-translational modifications, the detyrosination/tyrosination cycle, has the properties of affecting of structural and dynamic functions of microtubules that can control cell division and cell elongation. According to the same role of ABA and the detyrosination/tyrosination cycle to regulate plant growth, we would like to investigate the effect of ABA on plant growth through the detyrosination/tyrosination pathway. So, we observed the growth of roots and coleoptiles and analyzed the change of detyrosinated α-tubulin and tyrosinated

α-tubulin in rice plants overexpressing TUB6-GFP in the presence of ABA. These phenotypes we observed showed ABA inhibition of plant growth. Western blot analysis demonstrated the function of ABA on plant growth by regulating detyrosinated α-tubulin.

6. outlook

The tyrosinated α-tubulin should be producted directly by a TTL, while the TTC yields detyrosinated α-tubulin (Kumar and Flavin, 1981; Gundersen*et al*., 1987). According to these binding characteristics, where TTL and TTC prefer to bind to tubulin dimers and assembled microtubules, respectively, it leads to the implication that stable microtubules should accumulate detyrosine α-tubulin, while dynamic microtubules contain more tyrosine α-tubulin. However, in our present work, the application of Mas7 yielded more tyrosinated α-tubulin not detyrosinated α-tubulin. It has tested that Mas7 can stimulate G-protein to activate PLD activity to produce PA which can recruit MAP65-1 protein to stabilize MTs (Dhonukshe *et al*., 2003; Zhang *et al*., 2022) . Morever, before, in our lab, it also demonstrated that OsTTLL12 competes the C-terminal binding sites of α-tubulin with MAP65-1 resulting in preventing the couples of microtubules and MAP65-1 protein (Zhang *et al*., 2022) Therefore, we assumed such a hypothesis that when Mas 7 activated PLD activity to produce more PA, which could bind to more MAP65-1 to stabilize microtubules and induce more detyrosinated α-tubulin. However, there was a contradiction with the results we got, where the application of Mas7 not only disassembled microtubules but also decreased detyrosinated α-tubulin level . For this situation, We guessed there is one component that might have two faces: OsTTLL12 was, in our hands, a tubulin detyrosinase. On the other hand, there seemed to be no other candidate for a ligase. This protein may play both jobs, depending on association with other partners. In future, in order to know the function of OsTTLL12 protein, we will purify this protein to do a pulll-down experiment. Then, we will try to get the protein identified.

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Appendix

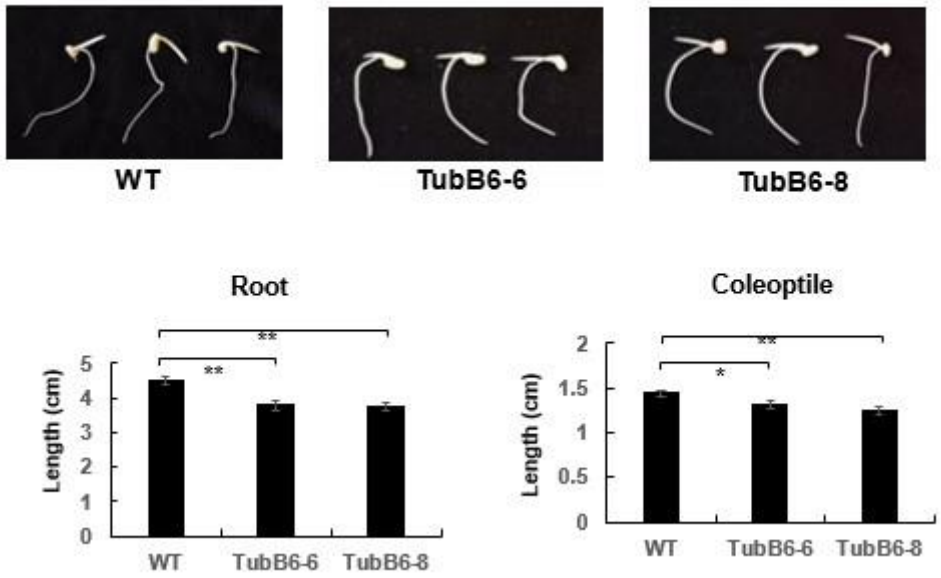

Fig. S1. Phenotype of TubB6-6 and TubB6-8 rice seeding compared to WT. A, B and C, representative image of root and coleoptile of wildtype, TubB6-6 line or TubB6-8 at day 5 after germination. D and E. Roots and coleoptiles length of wildtype, TubB6-6 and TubB6-8 lines. Differences were tested for significance with a Student's t-test for paired data with * indicating significant differences at P <0.05 and ** at P <0.01. Data is from three biological replicates and each replicate represents 7 seedlings.

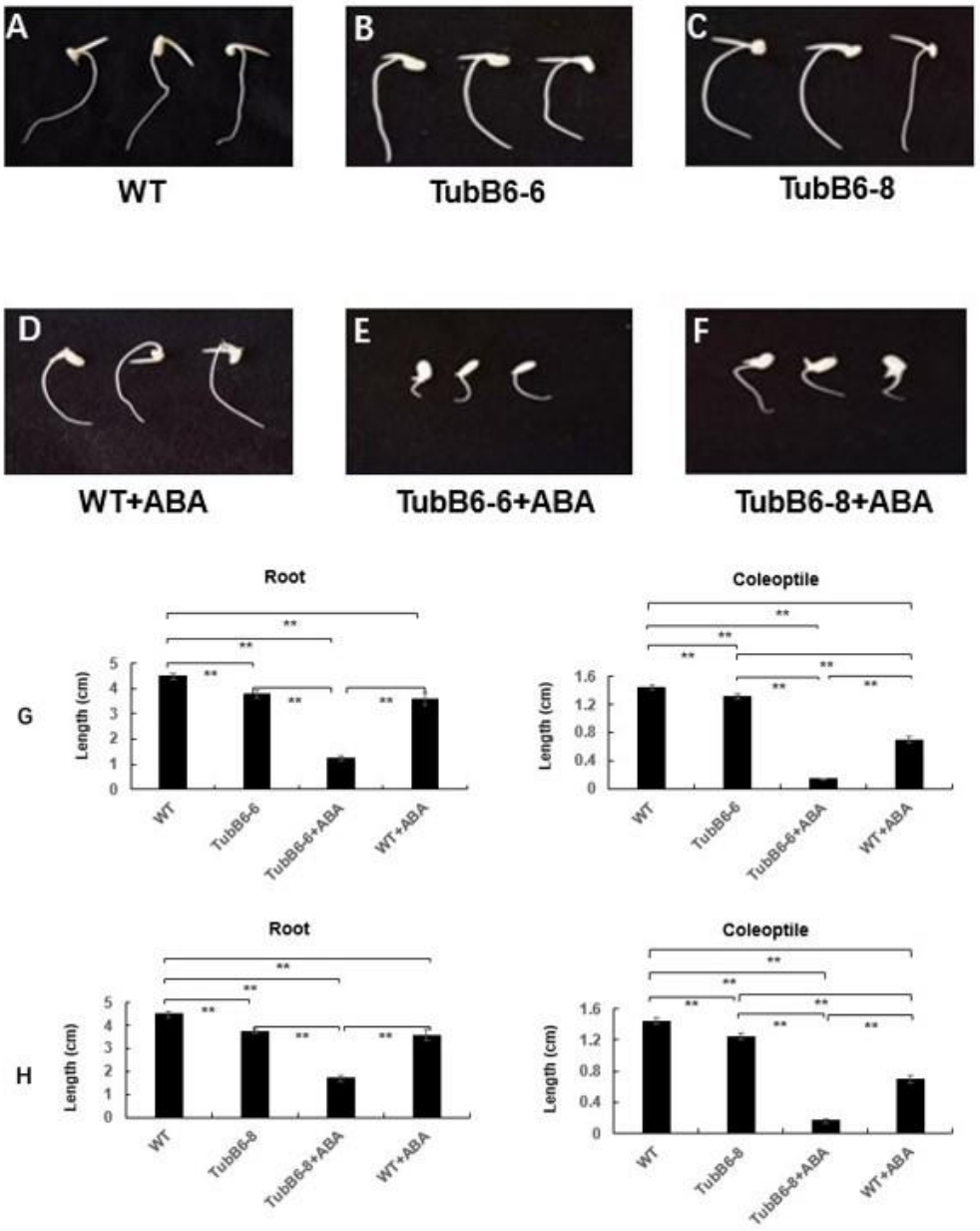

Fig. S2. Phenotype of TubB6-6 and TubB6-8 rice seeding compared to WT at day 5 after germination. A, B and C, representative image of root and coleoptile of wildtype, TubB6-6 line or TubB6-8. And D, E and F representative image of root and coleoptile of wildtype, TubB6-6 line or TubB6-8 in presence of ABA treatment. G and H. Roots and coleoptiles length of TubB6-6 and TubB6-8 lines compared to wildtype either in the absence or presence of ABA treatment. Differences were tested for significance with a Student's t-test for paired data with * indicating significant differences at P <0.05 and ** at P <0.01. Data is from three biological replicates and each replicate represents 7 seedlings.

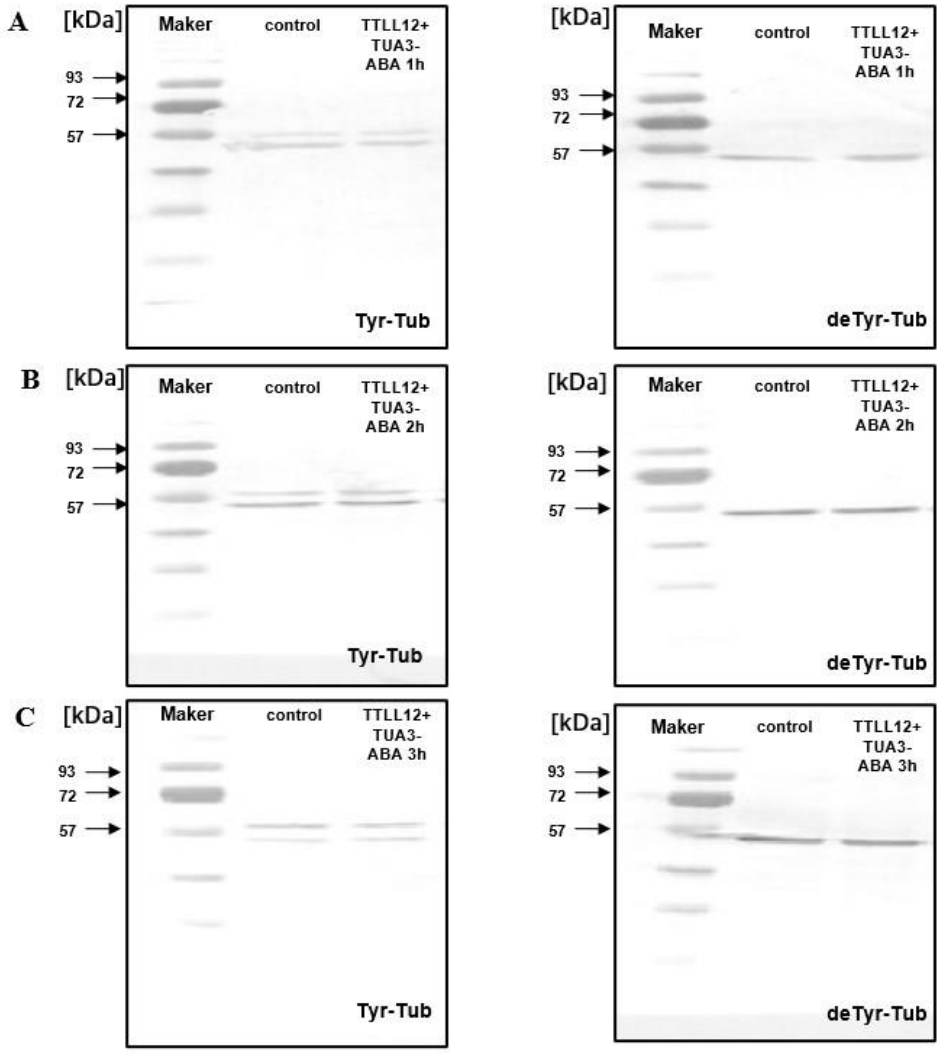

Fig. S3. Response in the post-translational modification of a-tubulin to ABA in BY-2 cells co-expressing *NtTUA3-GFP* and *OsTTLL12-RFP*. A, B and C. Representative Western blots after probing with monoclonal antibodies against tyrosinated a-tubulin (tyr-tub) and detyrosinated a-tubulin (detyr-tub) for 1 h, 2h and 3 h of treatment with 50 μM ABA as compared to control cells incubated without ABA (con). The arrowhead in 57kDa indicates tyrosinated/detyroinated form of α-tubulin.

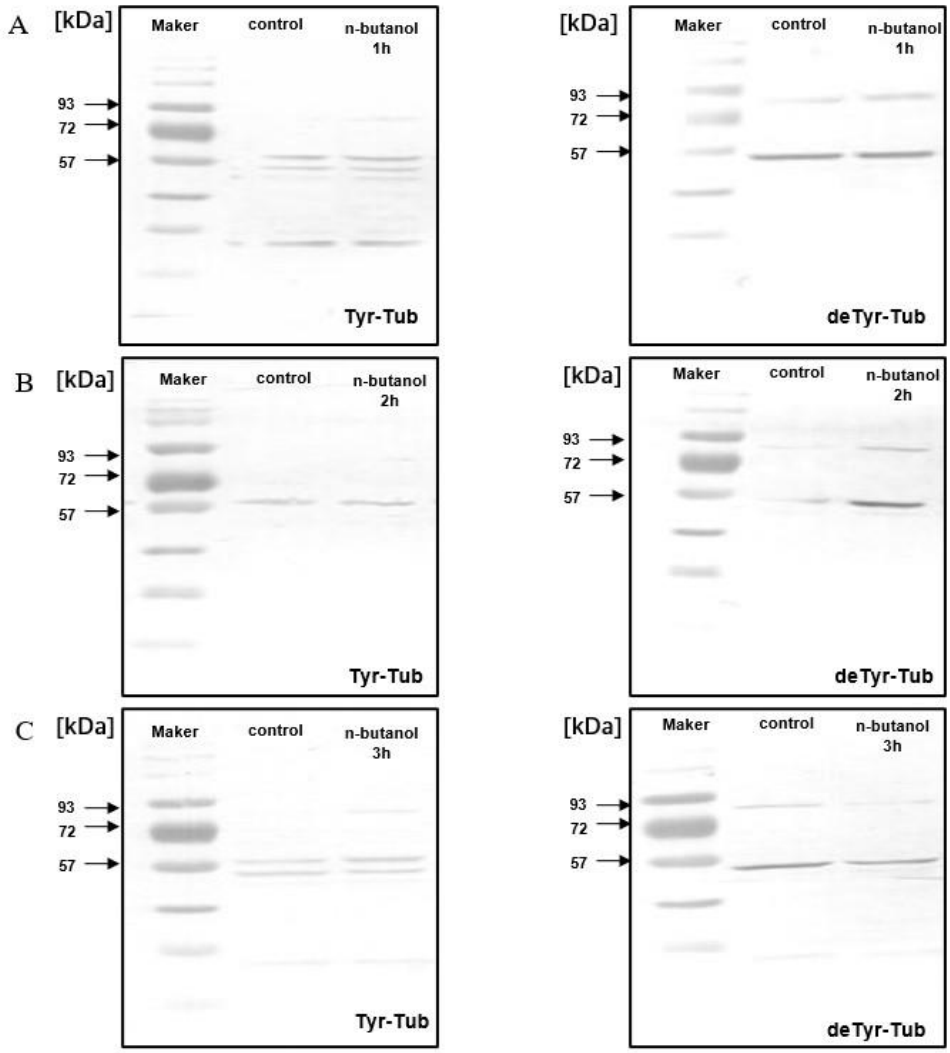

Fig. S4. Response in the post-translational modification of a-tubulin to ABA in BY-2 cells expressing the *NtTUA3-GFP* marker. A, B and C. Representative Western blots after probing with monoclonal antibodies against tyrosinated a-tubulin (tyr-tub) and detyrosinated a-tubulin (detyr-tub) for 1 h, 2h and 3 h of treatment with 50 μM ABA as compared to control cells incubated without ABA (con). The arrowhead in 57kDa indicates tyrosinated/detyroinated form of α-tubulin.

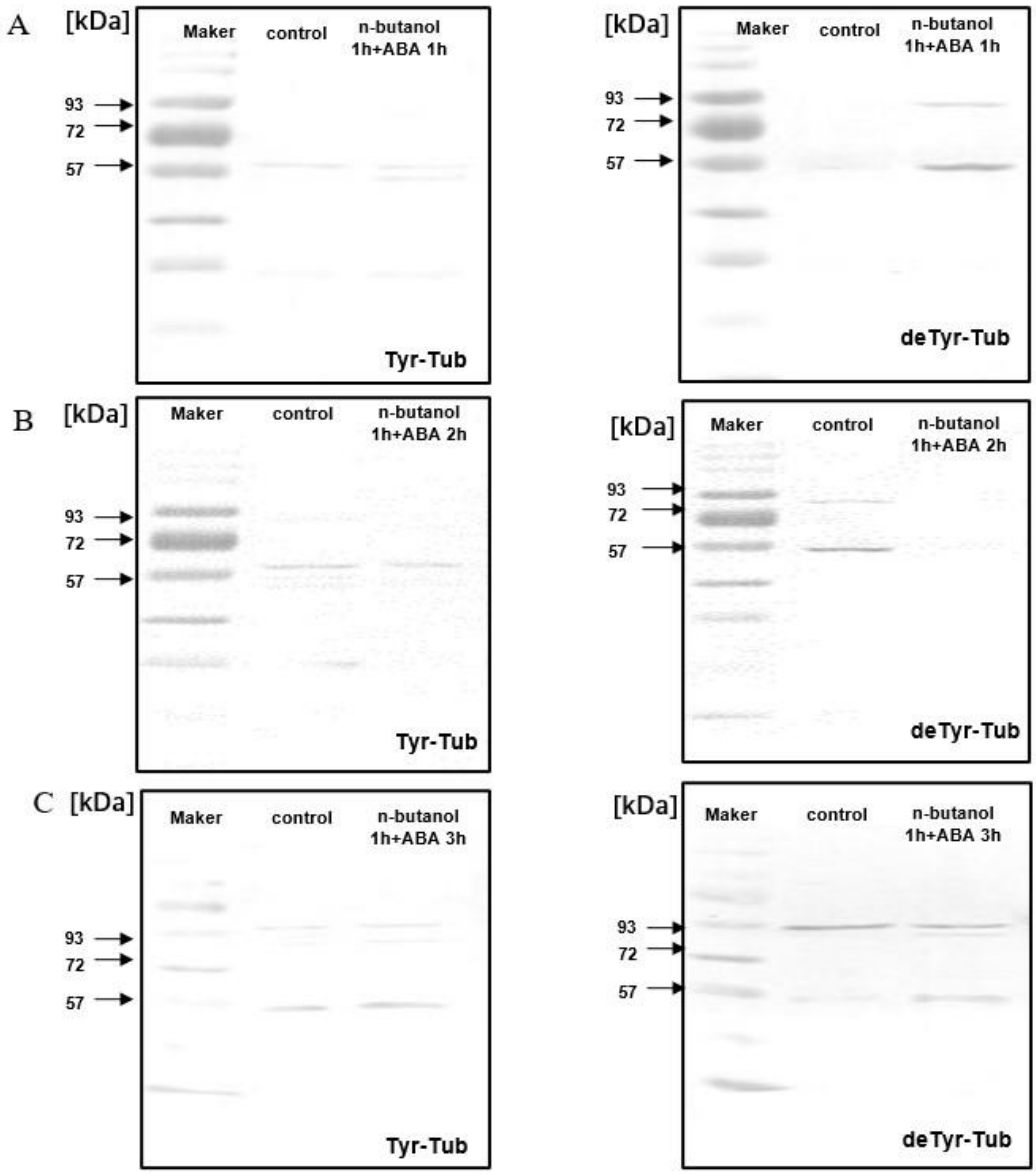

Fig. S5. Response in the post-translational modification of a-tubulin to ABA in BY-2 cells expressing the *NtTUA3-GFP* marker. A, B and C. Representative Western blots after probing with monoclonal antibodies against tyrosinated a-tubulin (tyr-tub) and detyrosinated a-tubulin (detyr-tub) for 1 h, 2h and 3 h of treatment with 50 μM ABA after pretreatment with n-butabol for 1h. The control cells incubated without double ABA and n-butanol treatments . The arrowhead in 57kDa indicates tyrosinated/detyroinated form of α -tubulin.

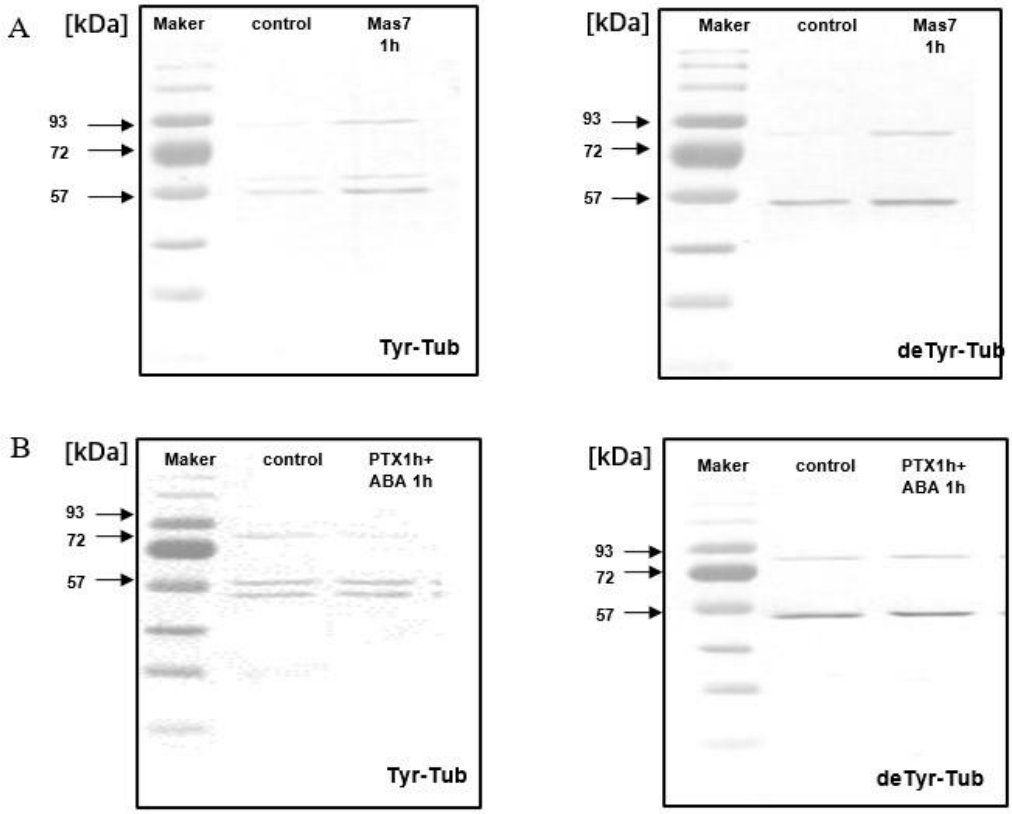

Fig. S6. Response in the post-translational modification of a-tubulin to ABA in BY-2 cells expressing the *NtTUA3-GFP* marker. A. Representative Western blots after probing with monoclonal antibodies against tyrosinated a-tubulin (tyr-tub) and detyrosinated a-tubulin (detyr-tub) for 1h of the G-protein activator Mastoparan 7 (Mas7, 5 μM). B. Representative Western blots after probing with monoclonal antibodies against tyrosinated a-tubulin (tyr-tub) and detyrosinated a-tubulin (detyr-tub) for 1h of treatment with 50 μM ABA after after pretreatment for 1 h with the G-protein inhibitor Pertussis Toxin (PTX, 10 $\mu g \cdot mL^{-1}$). The arrowhead in 57kDa indicates tyrosinated/detyroinated form of α-tubulin.