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β-AMINOBUTYRIC ACID (BABA): TOWARDS UNDERSTANDING ITS BIOSYNTHESIS, LOCALIZATION AND HORMONAL REGULATION IN *Arabidopsis thaliana*

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Abstract and keywords

Priming is defined as a physiological state induced by a priming stimulus that allows a plant to deploy a more rapid and more robust defense response to stresses compared with a non-primed plant. β-aminobutyric acid (BABA) has emerged as one of the best stimuli to study priming. Plants can synthesize BABA and accumulate it after being exposed to both biotic or abiotic stress. The plant immune system regulates BABA accumulation during pathogen infection. BABA concentrations vary depending on organ type and with the developmental stage. Flowers, senescent leaves and seeds are the sites of major accumulation. Interestingly, the early senescence and constitutive priming mutant *cpr5-2* shows higher basal and induced BABA concentrations compared to its wild type.

Besides *cpr5-2*, no other mutants related to BABA have been characterized up to now. Therefore, we performed RNA-seq analysis to identify common genes expressed during various BABA-inducing biotic and abiotic stresses in Arabidopsis Col-0 and *cpr5-2*. The analysis revealed ten genes up-regulated in common and one down-regulated gene. Nevertheless, T-DNA insertional lines of the up-regulated genes did not show a wild-type BABA concentration after salt stress application, keeping unsolved the search for genes involved in BABA metabolism in plants.

Furthermore, we are looking at the relation between BABA and plant hormones. Exposition of Arabidopsis to different plant hormones revealed that only ABA led to an increase of the BABA concentration. The bioactive form of ABA, (+)-ABA, showed a more robust BABA induction phenotype. Mutants of the three *SnRK2* genes, key regulators of ABA signaling, showed BABA induction after (+)-ABA application, suggesting a compensation effect.

Finally, to get further information on BABA localization in plants, BABA has been modified chemically, adding an alkyne tag. Successively, a copper-catalyzed azide-alkyne cycloaddition (CuAAC) between tagged BABA and an azide Alexa fluor® was performed, generating fluorescence tagged BABA molecules. These molecules were then visualized on confocal microscopy, showing a cell wall localization of BABA in Arabidopsis roots and globular subcellular structures. Leaves showed no labeling. However, the results are too early to define precisely the exact localization of BABA in plants.

Keywords: BABA, Arabidopsis, RNA-seq, Biotic stress, abiotic stress, Phytohormones, ABA, SnRK2s, CuAAC, confocal microscopy.

Résumé et mots-clés

Lorsqu'elles exposées à des évènements stressants de basse intensité ou stimulées par des molécules spécifiques, les plantes peuvent répondre plus rapidement et vigoureusement aux stress. Ce phénomène est appelé *priming*. Parmi les molécules qui l'induisent, l'acide β-aminobutyrique (BABA) peut être considéré comme l'une des mieux caractérisées. Les plantes synthétisent du BABA et augmentent leurs concentrations lors d'un stress, biotique ou abiotique. Cependant, sa biosynthèse, la régulation hormonale et sa localisation subcellulaire sont peu, voire pas encore connues. L'objectif de cette thèse a alors été de clarifier les aspects plus méconnus de la physiologie de BABA chez *Arabidopsis thaliana*.

Pour comprendre le métabolisme de cette molécule, une analyse RNA-seq a été réalisée en comparant après un stress, les transcrits des Arabettes avec ceux du mutant *cpr5-2*, possédant des concentrations basales plus hautes que le type sauvage. Cette analyse a permis de trouver dix gènes surexprimés et un gène sousexprimé en commun. Cependant, la détermination des concentrations de BABA, chez des lignées T-DNA des gènes surexprimés, n'a pas permis de sélectionner un ou plusieurs mutants, dont les niveaux étaient altérés par un stress abiotique.

Pour avoir une connaissance plus précise des signaux hormonaux contrôlant l'accumulation de BABA chez les plantes, des Arabettes ont été traitées de deux différentes manières en employant plusieurs solutions de phytohormones. Seule l'injection dans le sol de l'acide abscissique (ABA) a augmenté les niveaux de BABA, alors que sa forme bioactive (+)-ABA provoquait ce phénomène, tant en l'injectant dans le sol qu'en le sprayant. Pour lier cette augmentation avec la voie de signalisation de ABA chez Arabidopsis, deux mutants des trois SnRK2 ont été testé. Pour deux mutants s*nrk2.2 snrk2.3* et *snrk2.6*, les concentrations de BABA se sont révélées comparables au type sauvage. Ce phénotype dépendrait donc probablement des effets de compensation entre les trois kinases.

Pour localiser subcellulairement BABA, la microscopie confocale a été utilisée *in vivo* sur des Arabettes. Pour cela, une étiquette avec un alcyne a été ajouté à BABA (BABA-TAG), ne provoquant pas une diminution totale de sa capacité d'induction de résistance. La visualisation de BABA s'est faite via une réaction catalysée par le Cu(I) permettant d'attacher à l'alcyne un groupement azoture fluorescent. Après deux jours d'incubation, BABA montrait une localisation pariétale et se plaçait dans des structures globulaires intracellulaires. Cependant, d'autres expériences sont nécessaires pour définir correctement sa localisation chez les plantes.

Mots-clés : BABA, Arabidopsis, RNA-seq, stress biotique et abiotique, phytohormones, ABA, SnRK2, CuACC, microscopie confocale.

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1. General introduction.
The priming agent BABA: from inducer of resistance to plant metabolite

Francesco Stefanelli and Brigitte Mauch-Mani

1.1. Plant immunity and stress tolerance

1.1.1. Constitutive defenses

Plants have to face a wide range of attackers, such as viruses, bacteria, fungi or oomycetes. Animals like nematodes or insects also represent a danger for plant health (Agrios, 2005). The epidermis is the first barrier to dodge such a variety of enemies. In fact, in the aerial part of the plant, epidermal cells synthesize a waxy cuticle layer that represents a difficult barrier to overcome, as many pathogens lack of cuticle-degrading enzymes (Freeman and Beattie, 2008). Furthermore, specialized epidermal cells, such as trichomes and guard cells, protect plants from a pathogen attack. These barriers not only protect plants from biotic stress but also play an important role in abiotic stress, notably in water loss (Freeman and Beattie, 2008). Alongside, plants dispose of a vast arsenal of constitutive defense molecules belonging to different chemical families, collectively called phytoanticipins. Phytoanticipins have a strong antimicrobial activity and can be considered *bona fide* plant antibiotics (VanEtten et al., 1994; Piasecka et al., 2015). However, pathogens evolved techniques to pass these first lines of defenses and to reach the internal tissues of plants.

1.1.2. Local immunity

In contrast to animals, plant tissues do not host mobile specialized immune cells. However, plants evolved both innate immunity and acquired resistance to fight pathogens (Jones and Dangl, 2006; Conrath et al., 2006). Once they reach a plant cell, pathogens are perceived by membrane-anchored receptors, commonly named pattern-recognition-receptors (PRRs). PRRs possess an extracellular domain that perceives pathogen-associated molecular patterns (PAMPs), which are evolutionary conserved and microbe-unique domains of pathogens (Macho and Zipfel, 2014). The perception of PAMPs triggers then the so-called PAMP-triggered immunity (PTI) (Jones and Dangl, 2006; Boller and Felix, 2009). A classic example of a PAMP is the 22 amino acids bacterial flagellin epitope, flg22, specifically perceived and bound by the membrane receptor FLS2. After the binding of flg22, FLS2 forms a heterodimer with the co-receptor BAK1, leading to the downstream activation of PTI (**Fig 1-1**) (Gómez-Gómez et al., 1999; Gómez-Gómez and Boller, 2000; Chinchilla et al., 2006; Sun et al., 2013). Damage-associated molecular patterns (DAMPs), such as the small Atpep peptides or the cell

wall fragments olygogaracturonides, can activate PTI as well (D'Ovidio et al., 2004; Huffaker et al., 2006; Huffaker and Ryan, 2007).

Although PTI is sufficient in halting a microbial attack, some pathogens evolved the ability to avoid PTI and to block the immune response injecting effector proteins (formerly known as avirulence (Avr) proteins) into the plant cell (Flor, 1971; Jones and Dangl, 2006). In an evolutionary "arms race", plants evolved intracellular resistance (R) proteins, which detect effectors and trigger a more robust form of PTI called effector-triggered immunity (ETI) (Jones and Dangl, 2006; Thomma et al., 2011). The larger part of *R* genes encodes the nucleotide-binding and leucine-rich repeat (NB-LRR) domain protein family. NB-LRRs are divided, in turn, into the two TIR-domain-containing (TNL) and CC-domain-containing (CNL) subfamilies (McHale et al., 2006). A representative case of ETI occurs in the pathosystem Arabidopsis accession Col-0/*Pseudomonas syringae* pv tomato (*Pst*) DC3000 carrying the Avr gene *Rpt2* (AvrRpt2), where the R protein RPS2 recognizes the effector Rpt2, starting then the immune response (**Fig. 1-1**) (Whalen et al., 1991; Kunkel et al., 1993; Mindrinos et al., 1994).

During both PTI and ETI, plants deploy a wide set of responses against attackers. These responses are similar but vary in amplitude, suggesting that ETI may be a stronger form of PTI, and this dichotomy may be obsolete (Dodds and Rathjen, 2010; Thomma et al., 2011).

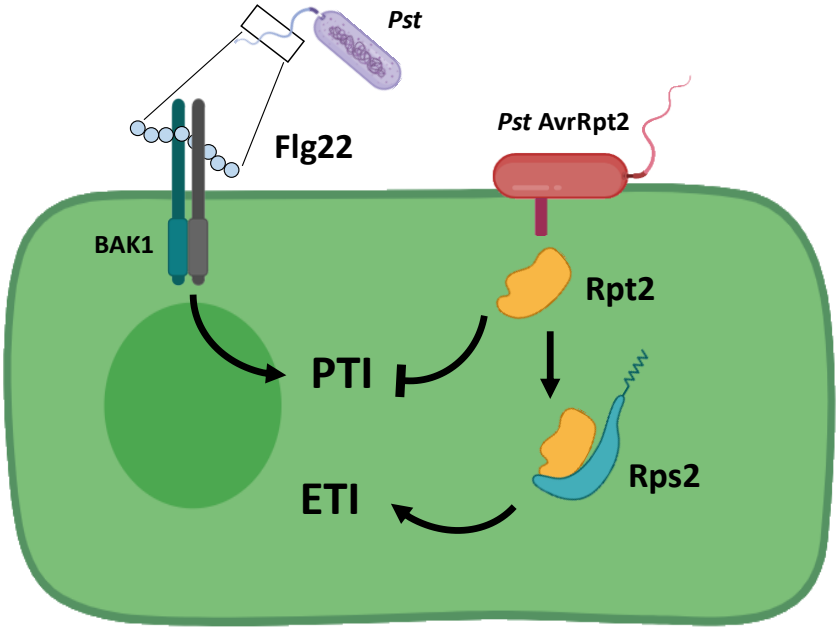

Fig. 1-1: PTI and ETI in the *Pseudomonas syringae*/*Arabidopsis thaliana* pathosystem. The dimerization of BAK1 with the FLS2 receptor, after the perception of PAMP flg22 of *Pst*, leads to the establishment of PTI. The strain *Pst* AvrRpt2 injects the effector Rpt2, which is able to block PTI. However, the Arabidopsis ecotype Col-0, possess the NB-LRR receptor Rps2 that can harbor Rpt2 and set up ETI.

1.1.3. Molecular and biochemical events during local immunity

Pathogen perception leads to the rapid synthesis of reactive oxygen species (ROS), such as hydroxyl radical (OH•), superoxide ($O2•^-$), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2) (Wojtaszek, 1997a). ROS not only play a role in plant defense but also participate to several aspects of plant physiology, such as stomatal closure, growth, cell expansion, and abiotic stress tolerance, and work as signaling molecules as well. One of the ROS signals is the gaseous molecule nitric oxide (NO), which works as a diffusible messenger in cell-cell communication (Wrzaczek et al., 2013; Domingos et al., 2015). Peroxidases (POXs) and NADPH oxidases are the main enzymatic families involved in the extracellular ROS production. Peroxisome and plastids are instead the main sites of ROS endocellular production (Kawano, 2003; Sagi and Fluhr, 2006). POX is involved in ROS production, antimicrobial phenolic synthesis, and may contribute to lignification as well (Brisson et al., 1994; Wojtaszek, 1997b; Bestwick et al., 1998).

Along with ROS synthesis, during both PTI and ETI, an increase in cytosolic Ca^{2+} concentrations occurs (Zhang et al., 2014). Released Ca^{2+} ions bind afterward a class of Ca^{2+}-dependent protein kinases (CDPKs), which in turn phosphorylate different substrates and activates hormonal signaling, ROS burst and the transcription of defense-related genes (Romeis et al., 2001; Boudsocq et al., 2010; Schulz et al., 2013). Although the significant roles played by CDPKs, the majority of biotic stress responses depend on the mitogen-activated protein kinases (MAPKs) signaling cascade. MAPKs are members of a serine/threonine-protein kinase family that activate several cytoplasmic or nuclear targets upon different environmental or developmental stimuli (Jagodzik et al., 2018). Among the targets of MAPKs, there are WRKY transcription factors. WRKYs regulate the transcription of several defense-related genes, the different phytoalexins biosynthetic pathways (Chen et al., 2019). Phytoalexins are antimicrobial molecules with a low molecular weight whose biosynthesis increases after infections. Phytoalexins belong to different chemical families, such as phenylpropanoids and sesquiterpenes. Some phytoalexins can work as phytoanticipins as well (Hammerschmidt, 1999).

A characteristic of ETI is programmed cell death, provoking necrotic lesions around the site of pathogen penetration, confining thus the pathogen and interrupting its spread. This phenomenon takes the name of hypersensitive response (HR) and is characterized by a severe ROS burst (Levine et al., 1994; Dangl et al., 1996; Morel and Dangl, 1997; Heath, 2000; Mur

et al., 2007). Furthermore, cells surrounding HR-necrotic lesions trigger the expression of pathogenesis-related (PR) proteins and other defense-related genes (Somssich et al., 1988; Schmelzer et al., 1989). PR proteins are a class of antimicrobial proteins inducible by plant pathogens or defense-related signals. Seventeen families compose the PR proteins class, clustered by their sequence similarities, biological function, and enzymatic structure (Ali et al., 2018). PR-1 was discovered already 50 years ago and has become the classical marker in plant immunity, as being the most abundant PR protein. *PR-1* gene codes for a sperm coating protein family able to sequestrate sterols. Hence, PR-1 is efficiently active in the defense against sterol-auxotroph oomycetes, such as *Phytophthora* (Gianinazzi, 1970; Van Loon and Gerritsen, 1989; Gamir et al., 2017). Other PR proteins function as peroxidases, defensins, glucanases, and chitinases, and are effective against a plethora of phytopathogens (Van Loon and Van Strien, 1999).

An important aspect of HR is the fortification of the primary cell wall in surrounding cells, through the apposition of polymers, such as callose and lignin (Bellincampi et al., 2014). On one side, callose is a (1,3)-β-glucan polysaccharide with multifaceted roles in plant physiology, including response to biotic stress (Chen and Kim, 2009). Callose accumulation can occur in plasmodesmata to shrink the plasmodesmal pore and results important in the defense against viruses (Chen et al., 2000). Flg22 application also induces callose accumulation (Clay et al., 2009). However, plants accumulate callose in cell wall thickening structures named “papillae” found around the HR-related necrosis or at the site of pathogen penetration (Bellincampi et al., 2014; Voigt, 2014). Besides, papillae are a site of ROS and other antimicrobial compounds accumulation (Aist, 1976; Thordal-Christensen et al., 1997; Celio et al., 2004; Bellincampi et al., 2014). Callose constitutes the main component of papillae, although pectins, cellulose, hemicellulose, and structural protein, glycoproteins, and lignin also occur. Lignin is instead a phenolic polymer, a major component of secondary cell walls, particularly in xylem, where provides rigidity and strength to the other polysaccharide components (Higuchi, 1985). The accumulation of lignin and lignin-like phenols occurs in incompatible interactions with bacteria (Reimers and Leach, 1991). Lignification is also particularly efficient in contrast against fungi and other penetrating pathogens, such as nematodes, blocking them at the site of the attempted penetration (Moerschbacher et al., 1990; Lange et al., 1995; Kawasaki et al., 2006; Dhakshinamoorthy et al., 2014). Lignification in the papillae impedes the progress of the penetration peg of fungi into the cell and strengthens the cell wall against the appressorium pressure (Bechinger et al., 1999).

1.1.4. Phytohormones signaling in local immunity

An important aspect of plant-pathogen interaction is the boosting of different hormonal pathways. A hormone with a newly recognized role in plant resistance is abscisic acid (ABA) (Fan et al., 2009; Ton et al., 2009). ABA has been generally associated with a negative role in plant-pathogen interaction, as different pathogens boost ABA signaling to increase host susceptibility and ABA has an antagonistic effect on other hormonal pathways (Anderson et al., 2004; Ton et al., 2009). However, different studies have demonstrated the involvement of ABA in the pre-invasive and early post-invasive stages of a pathogen attack (Ton et al., 2009). Indeed, ABA is a key phytohormone involved in stomatal closing, particularly during osmotic stress (Fernando and Schroeder, 2016). Furthermore, ABA-mediated stomatal closure requires the biosynthesis of H_2O_2 and, in turn, of NO (Bright et al., 2006). Flg22 perception by FLS2 and lipopolysaccharides mediate NO production and consequently induce stomatal closing as well. The ABA-biosynthetic mutant *aba1-3* and the ABA-signaling mutant *ost1* are impaired in the flg22 and lipopolysaccharides-mediated stomatal closure. Also, the NO synthase inhibitor L-NNA impedes flg22- and lipopolysaccharide-mediated stomatal closure (Zeidler et al., 2004; Zipfel et al., 2004; Kim et al., 2005; Melotto et al., 2006). ABA also positively mediates the deposition of callose and papillae build-up, considering that both the ABA deficient mutant *aba1-3* and the ABA-insensitive mutant *abi1-1* are susceptible to *Leptospheria maculans* and show fewer callose deposits (Ton and Mauch-Mani, 2004; Kaliff et al., 2007). In contrast to *aba3-1*, the enhanced-ABA concentrations mutant *cds2-1D* shows increased resistance when infected with *Alternaria brassicicola* (Fan et al., 2009). Moreover, ABA application boosts callose deposition in response to both *A. brassicicola* and the necrotrophic pathogen *Plectosphaerella cucumerina* (Ton and Mauch-Mani, 2004; Flors et al., 2008).

ABA is not the only regulator of callose deposition. Ethylene (ET), one of the first plant hormones to be discovered, also seems to control callose deposition, as the ET receptor mutant *etr1-1* and the ET insensitive mutant *ein2-1* were impaired in flg22-induced callose deposition (Clay et al., 2009). Furthermore, ET controls ROS burst during PTI, and ET-signaling regulates the expression of the flagellin receptor *FLS2* (Mersmann et al., 2010). ET usually acts synergically with the phytohormone jasmonic acid (JA) (Bürger and Chory, 2019). ET and JA share the ERF transcription pathway-signaling cascade, which leads to the activation of the antimicrobial peptide defensin PDF1.2 (Penninckx et al., 1998; Bürger and Chory, 2019). Generally, JA and ET-signaling are activated during a necrotrophic pathogen or chewing insect attack (Baldwin et al., 1997). After wounding or mechanical damage, the biosynthesis of JA,

JA-precursors and their derivatives, defined as jasmonates, begins from linoleic acid precursor (Wasternack, 2007). Plastidial phospholipase DAD1 releases linoleic acid from phospholipids. The lipoxygenase (LOX) afterward converts linoleic acid into OPDA and finally into JA. The JA-amidosynthetase (JAR1) converts JA into its active form, JA-Ile (Fonseca et al., 2009). JA-Ile sequestrates in turn JAZ proteins, negative regulators of the MYC transcription factors, which are positive activators of the JA-signaling response (**Fig. 1-2**). MYCs, however, work independently from the ERF pathway (Thines et al., 2007; Fernández-Calvo et al., 2011). JA and jasmonates activate several defense responses: alkaloid production, trichomes formation, and production of proteinase inhibitors, for example (Wasternack, 2007).

By contrast, salicylic acid (SA) is generally implied in defense against biotrophic pathogens (Bürger and Chory, 2019). Plants accumulate SA and SA-conjugates after pathogen perception (**Fig. 1-2**) (Métraux et al., 1990; Enyedi et al., 1992). SA is mainly synthesized via the isochorismate pathway. Chorismate is firstly converted into isochorismate by isochorismate synthase (ICS). Then ICS is converted into SA (Wildermuth et al., 2001; Rekhter et al., 2019; Torrens-Spence et al., 2019). The members of the Non-expressor of Pathogenesis-Related Genes (NPR) protein family are the main receptors involved in SA-perception, and among them, NPR1 is the main receptor and activator of SA-signaling (Fu et al., 2012; Wu et al., 2012; Ding et al., 2018). SA-binding to cytoplasmic NPR1 complexes leads to a monomerization of NPR1. NPR1 monomers later translocate into the nucleus where they interact with TGA transcription factors, boosting then defense response (**Fig. 1-2**) (Zhou et al., 2000; Tada et al., 2008). SA is implicated in the formation of the HR and in the activation of PR proteins, with PR-1 as a marker of the SA-defense signaling (**Fig. 1-2**) (Dorey et al., 1997; Cameron et al., 1999a; Fu et al., 2012). Furthermore, SA application alone can increase PR protein concentrations (White, 1979; Van Loon and Antoniw, 1982). Plants accumulate SA even during PTI, considering that the SA-biosynthetic mutant *sid2* is impaired in expressing PTI after PAMP perception (Tsuda et al., 2008). The crosstalk between JA- and SA-pathways is generally antagonistic, as several studies demonstrated a mutual inhibitory effect (Glazebrook, 2005). At the same time, SA-signaling interplays with the JA-pathway to coordinate the local defense response, suggesting a simultaneous induction of the two pathways (Tsuda et al., 2009). This contradiction was explained by a different spatiotemporal activation of the two pathways around the HR necrosis, as SA-signaling is more activated on the inner layer of cells around the dead zone, while JA-signaling in the outer one. This activation of JA-signaling may attenuate an overflow of the SA-response (Betsuyaku et al., 2018).

1.1.5. Systemic immunity

From the site of pathogen attack, plants send signals to uninfected tissues, activating then an immunity against possible future attacks. The activation of ETI, for example, is often associated with the onset of another form of immunity, which is triggered in distal uninfected tissues, the so-called systemic acquired resistance (SAR) (**Fig. 1-2**) (Fu and Dong, 2013). SAR can be activated not only by avirulent pathogens. The application of synthetic molecules, such as 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole S-methyl ester (BTH), also activate SAR (Uknes et al., 1992; Cameron et al., 1999b). SAR starts with the biosynthesis of SA (Klessig et al., 2018). SA is the main molecule involved in the induction of SAR, considering that the SA-biosynthetic mutant *ics1* fails to express SAR (Wildermuth et al., 2001; Klessig et al., 2018). Plants accumulate SA also in distal tissues, and SA-distal accumulation is necessary for SAR (**Fig. 1-2**) (Malamy et al., 1990; Métraux et al., 1990; Rasmussen et al., 1991; Gaffney et al., 1993). The *fmo1* mutant, which is impaired in SA-distal accumulation, is also impaired in SAR (Mishina and Zeier, 2006). However, the accumulation in systemic leaves does not depend on a SA translocation from the local leaf (Rasmussen et al., 1991). Other signaling molecules behave as mobile signals for SAR. The main candidates as signaling molecules for SAR are azelaic acid, glycerol-3-phosphate, amino acid pipecolic acid or the terpenic molecule dehydroabietinal (Malamy et al., 1990; Métraux et al., 1990; Lawton et al., 1995; Park et al., 2007; Jung et al., 2009; Chanda et al., 2011; Chaturvedi et al., 2012; Bernsdorff et al., 2016). However, the methylated form of SA, methyl salycilic acid (MeSA), was the first SAR signaling molecule to be identified (**Fig. 1-2**) (Park et al., 2007; Klessig et al., 2018). SA is firstly converted into MeSA by the methylesterase SABP2. Successively, MeSA moves to the distal uninfected tissue s where it is reconverted in SA and, in turn, activates NPR1, boosting thus the immune response (**Fig. 1-2**) (Cao et al., 1994; Glazebrook et al., 1996). SAR is characterized by the accumulation of PR proteins in the distal tissues to prevent secondary infections (Uknes et al., 1992). Successively, after a second pathogenic attack, SAR-treated plants potentiate the expression of PR proteins (Cameron et al., 1999b; Kohler et al., 2002). Therefore, PR proteins are considered as markers for the induction of SAR in distal tissues (Fu and Dong, 2013). Furthermore, SAR triggers enhanced callose deposition upon secondary infection, as *npr1-1* is impaired in the BTH-induced and primed callose deposition (**Fig. 1-2**) (Kohler et al., 2002). SAR confers immunity towards a vast range of attackers apart from the avirulent pathogen, triggering ETI. Besides, SAR maintains cell survival, and it is not associated with HR in distal tissues (Fu and Dong, 2013). Moreover, SAR confers immune

memory, which can last several weeks and can also be inherited to the progeny (Luna et al., 2012). These characteristics enable SAR to be considered as part of the priming phenomenon of plant immunity, which will be successively described (Conrath et al., 2002; Conrath et al., 2006).

Wounding and insect chewing provoke tissue damage, leading to an increase in JA concentrations (Baldwin et al., 1997). Subsequently, JA moves through the phloem and activates defense response in uninfected tissues (**Fig. 1-2**). Wounded scions of the tomato JA-insensible mutant *jai-1* are impaired in the expression of *proteinase inhibitor II* transcripts, although the wild-type rootstock retains this expression (Li et al., 2002). Furthermore, JA can also be converted into the methylated form MeJA, which acts as a volatile signal in inducing systemic defense (Seo et al., 2001). This form of immunity is called herbivore-induced resistance (HIR) (Pieterse et al., 2014). HIR comprises the systemic synthesis of proteinase inhibitors, alkaloids, and also volatiles in the uninfected leaves. ABA-dependent callose deposition is also boosted during HIR (Heil and Ton, 2008; Vos et al., 2013).

Along with SAR and HIR, a form of acquired immunity not dependent on pathogens or insects has been known for thirty years, the so-called induced systemic resistance (ISR) (Alstrom, 1991; Pieterse et al., 2014). ISR is the acquirement of a systemic immunity against a broad spectrum of pathogens and insect herbivores induced after root colonization by beneficial microbes (**Fig. 1-2**) (Pieterse et al., 2014). Among the mutualistic microbes involved in ISR can be included mycorrhizae, *Trichoderma*, *Pseudomonas*, and *Bacillus* (Pieterse et al., 2014). For the onset of ISR, beneficial microbes first suppress host immunity to properly colonize roots (Wang et al., 2012; Zamioudis and Pieterse, 2012). Afterward, they produce elicitors to establish ISR, such as 2,4-diacetylphloroglucinol or also volatile compounds (Weller et al., 2004; Pieterse et al., 2014). An important role in the activation of SAR in roots is played by ET-signaling and by the MYB72 transcription factor. Both root-specific ET-insensitive *eir1* and *myb72* knockout mutants are impaired in expressing ISR (**Fig. 1-2**) (Knoester et al., 1999; Alizadeh et al., 2013). Nevertheless, the mobile signal conferring immunity to systemic tissues in ISR is not known yet (Pieterse et al., 2014). Although similar in the establishment of resistance, ISR differs from SAR as PR proteins are not involved. ISR against *Fusarium oxysporium* induced by *Pseudomonas fluorescens* WCS417r, in Arabidopsis, and radish, do not require PRs and SA-signaling (Hoffland et al., 1995; Pieterse et al., 1996). ET and JA-signaling play a major role in the onset of ISR. Different biosynthetic or insensible mutants, such as *jar1* or *etr1*, are indeed impaired in *P. fluorescens* WCS417r-ISR (**Fig. 1-2**) (Pieterse et al., 1998;

Knoester et al., 1999; Pozo et al., 2008). Furthermore, transcripts of JA and ET-responsive genes, e.g., *PDF1.2*, accumulate during ISR (Van Oosten et al., 2008). An interesting common point between SAR and ISR is the central role played by NPR1, although not controlled by SA-signaling, but instead by JA/ET. Nevertheless, the role of NPR1 in ISR is not well understood (**Fig. 1-2**) (Spoel et al., 2003; Ramírez et al., 2010; Pieterse et al., 2012; Nie et al., 2017). Finally, during ISR, ABA-dependent callose deposition increases, conferring a wider spectrum of defense to primed plants (**Fig. 1-2**) (Van der Ent et al., 2009).

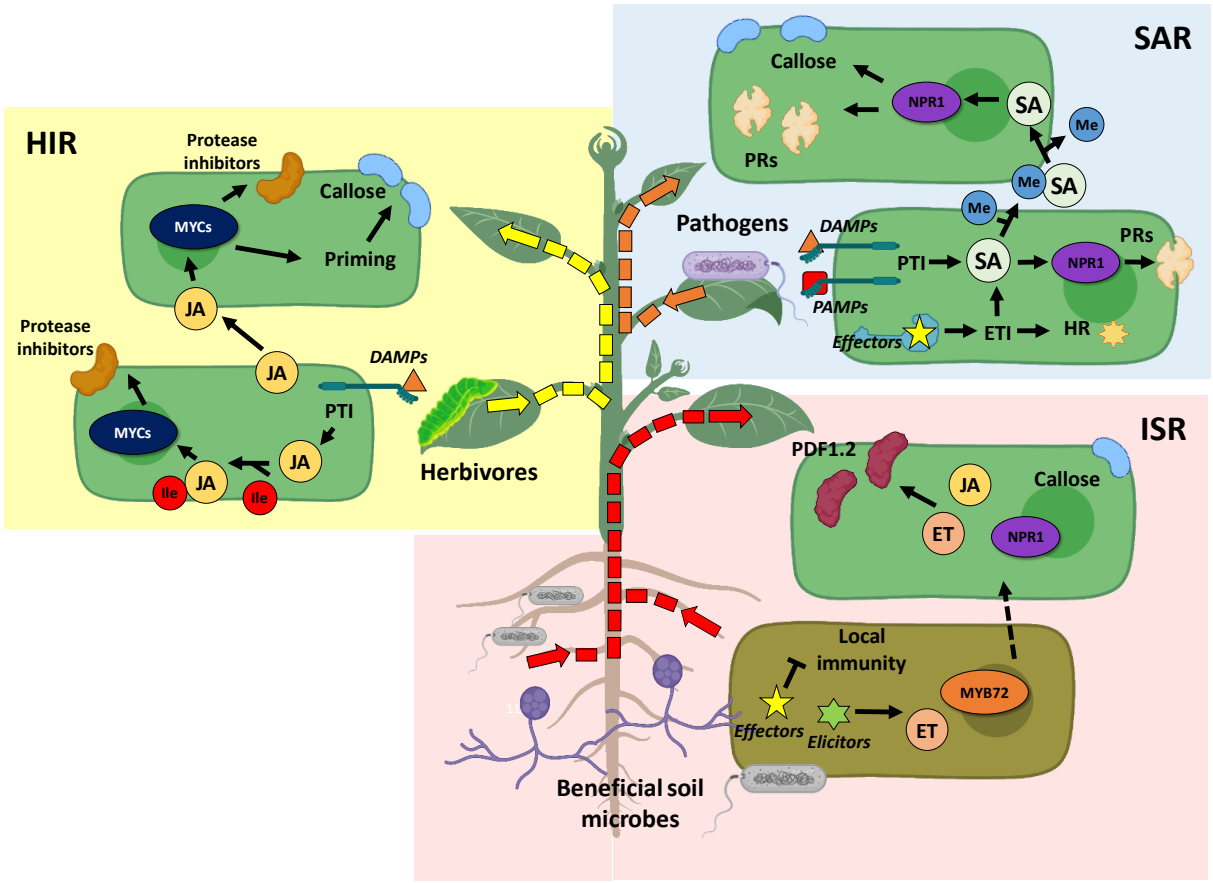

Fig. 1-2: The different types of systemic immunity in plants. **Herbivore induced resistance (HIR).** HIR starts after the attack of herbivores. It leads to the formation of DAMPs and the set-up of PTI. Then, the biosynthesis of JA and Jasmonates (e.g., JA-Ile) starts. These molecules activate MYCs transcription factors and the production of defense-related proteins (e.g., proteinase inhibitors). JA successively translocates into systemic tissues through the phloem, where it activates JA-defense related and priming responses, such as enhanced callose deposition. **Systemic acquired resistance (SAR).** SAR signaling starts when pathogen-derived stimuli, such as PAMPs, DAMPs, and effectors, establishing PTI and ETI. PTI and ETI increase ROS burst, activating then HR and the biosynthesis of SA. SA activates SA-dependent responses (e.g., *PR* genes induction), via the NPR1 receptor. To activate the systemic response, SA is methylated. MeSA moves to systemic tissues, where it activates the SAR response, such as priming for enhanced *PR* gene expression and callose deposition**. Induced systemic resistance (ISR).** Beneficial soil microbes block local root immunity through effectors, as done by pathogens. Furthermore, they inject elicitors that enhance ISR in roots via ET-signaling and MYB72 transcription factor. In systemic tissues, ISR is activated through the JA/ET signaling, which has a marker PDF1.2. A central role in ISR is played by NPR1, but in a different way from SAR. As occurring in the other form of systemic immunity, ISR also enhances callose deposition during a pathogen attack.

1.1.6. Age-related resistance

Many flowering plants express an age-related resistance (ARR) against different pathogens. ARR is correlated with the transition between developmental stages (Hu and Yang, 2019). A clear example comes from the pathosystem Arabidopsis Col-0/*Hyaloperonospora arabidopsidis* (*Hpa*) Emco5. Cotyledons of Arabidopsis Col-0 are susceptible, while true leaves are resistant to *Hpa* Emco5 (McDowell et al., 2005). Interestingly, the Wassilewskija (Ws-0) ecotype shows resistance at every development stage. This phenotype was due to a recessive mutation in the locus *RPP31*, whose function is still unknown (McDowell et al., 2005). ARR is also clearly evident in the rate of growth of *Pst* DC3000 in 4- and 7-week-old plants (Carella et al., 2015). ARR seems to depend on SA-signaling. Indeed, *NahG* transformed, and *npr1* mutant plants are impaired in adult leaf resistance in *Hpa* Emco5 infected plants (McDowell et al., 2005). Furthermore, SA accumulates with age in plants, while in SA-deficient mutants, SA-application restores ARR against *Pst* DC3000. The accumulation of SA confers resistance, also thanks to its antimicrobial activity (Wilson et al., 2017).

1.1.7. Plants dealing with abiotic stress

Plants not only face pathogens and herbivores but are also exposed to numerous abiotic stresses, which are caused by a lack or an excess of some environmental factors that plants need in an optimum range (Agrios, 2005). Abiotic stress provokes serious damages to plants. Osmotic stress, for example, reduces the uptake of water, generating oxidative stress, ion toxicity, nutrient imbalance, and liquid loss (Parida and Das, 2005). On another side, cold stress induces membrane disintegration, injuries, and solute leakage but can also induce dangerous effects as well as altering reproduction (Yadav, 2010). In contrast, high temperatures provoke inactivation of enzymes and protein denaturation and can lead to programmed cell death as well (Kampinga et al., 1995; Sangwan et al., 2002; Vacca et al., 2004). Finally, heavy metal accumulation leads to enzyme and protein damages, growth inhibition, oxidative stress, and inhibition of nutrient uptake, eventually causing plant death (Di Toppi and Gabbrielli, 1999).

However, plants evolved different strategies to contrast environmental stress. For example, glutathione works as a detoxifying molecule in response to heavy metal accumulation. The reduced glutathione levels mutant *cad2* shows hypersensitivity to both cadmium and copper (Cobbett et al., 1998; Xiang et al., 2001). Another example occurs during a heat shock when plants accumulate heat shock proteins (HSPs), which act as molecular chaperones to prevent

unfolded protein aggregation (Gurley, 2000). In opposite, during cold stress, plants modify their membrane lipid composition by increasing desaturated phospholipid content to prevent membrane rigidification (Anchordoguy et al., 1987). Furthermore, plants accumulate osmolytes and anti-freezing proteins that retain water and impede water loss (Lissarre et al., 2010). Similar acclimation processes also occur during osmotic stress. Typically accumulated osmolytes are sugars (e.g., trehalose, mannitol, or sorbitol) but also glycine betaine and particularly the amino-acid proline (Hasegawa et al., 2000; Dos Reis et al., 2012). Plants respond to osmotic stress by closing the stomatal pore, reducing water flow due to transpiration. As observed during pathogen penetration, stomatal closure during abiotic stress depends on ABA- and NO-signaling (García-Mata and Lamattina, 2001; Mustilli et al., 2002). ABA is a key hormone regulating distinct pathways during osmotic stress. The ABA-biosynthetic mutant *los5/aba3* shows reduced expression of stress-related genes upon osmotic and cold-stresses, and a diminished induction of proline during salt treatment (Xiong et al., 2001).

Interestingly, plants exposed to a previous mild abiotic stress can adapt their behavior towards a tougher event. This phenomenon is termed acclimation (Chinnusamy et al., 2007). For example, temperate plants can acquire tolerance to freezing temperatures by prior exposure to higher chilling temperatures. On another side, Arabidopsis can survive for 2 hours at 45 °C after being exposed to milder high temperatures for 90 minutes (Hong and Vierling, 2001; Chinnusamy et al., 2007). Beneficial microorganisms help plants in acquiring abiotic stress tolerance as well. For example, banana plantlets improve their salinity tolerance by a mutualistic relationship with mycorrhiza. Growth-promoting rhizobacteria induce the expression of stress-related genes and antioxidants, change the saturation pattern of phospholipids in membranes, and enhance the development of lateral roots (Yano-Melo et al., 2003; Dimkpa et al., 2009).

External chemical stimuli can also achieve abiotic stress tolerance. These stimuli can induce resistance to biotic stress as well, which is faster and stronger compared to non-treated plants, without elevated fitness costs. This phenomenon is named priming (Mauch-Mani et al., 2017).

1.1.8. Priming

Priming is defined as an enhanced sensitivity and responsiveness to stress resulting from a prior experience that leads to increased resistance and abiotic stress tolerance, more robust and faster in primed plants compared to a naïve plant (Mauch-Mani et al., 2017). Differently from priming, a defense response is still characterized by a faster, stronger, and sustained response to stress.Furthermore, the perception of the triggering stimulus is concomitant with the stress (Hilker et al., 2016; Martinez-Medina et al., 2016).

Priming is an integral part of ISR, SAR, and acclimation. However, priming has a minimal or no negative effect on plant energy resources, since the defense repertoire is deployed only after a challenge and not present in a constitutive manner (Balmer et al., 2015). Furthermore, primed plants show equal protection levels and suffer fewer fitness costs (van Hulten et al., 2006). Importantly, primed plants show a memory of the stimulus that is stored until the arrival of a challenge (Kandel et al., 2014; Conrath et al., 2015). If there is memory, a more robust response, better performance, and low fitness costs occur at the same time (**Fig. 1-3**). A priming response is also characterized by a broad spectrum of activity and low fitness costs (Martinez-Medina et al., 2016).

A plant is defined as primed upon the perception of an appropriate priming stimulus. In contrast, a plant is considered naïve that has not perceived any stimulus and does not display priming (Martinez-Medina et al., 2016). Priming stimuli are extremely varied and span from arbuscular mycorrhizae and plant-growth-promoting rhizobacteria and fungi, to stress itself or indicatives of imminent stress. Some chemical compounds also exhibit priming capacities (Martinez-Medina et al., 2016). Balmer et al. (2015) defined three different phases in priming: a priming phase, a following post-challenged primed-phase, and a transgenerational-priming phase. The priming phase starts with the perception of the priming stimulus getting the plant primed. The post-challenged primed-phase occurs when a challenge arrives and leads to an enhanced response. The transgenerational priming phase occurs in the progeny of primed plants, and seems to be mediated by epigenetic changes, such as hypermetilation of promoter of defense genes (Luna et al., 2012; Rasmann et al., 2012; Slaughter et al., 2012).

During the primed state, levels of PRRs increase, and enhanced expression of transcription factor genes (e.g., WRKYs) occurs. Accumulation of MAPKs also has been described (Beckers et al., 2009; Chavan and Kamble, 2013; Conrath et al., 2015). Interestingly, primed plants accumulate inactive forms of defense-related hormones, phytoanticipins, and indole

glucosinolates. A priming stimulus can lead to an increase of primary metabolism compounds, both sugars and amino acids (Chavan and Kamble, 2013; Gamir et al., 2014; Balmer et al., 2015). Priming causes epigenetic changes in the plant. For example, di-/tri-methylation and acetylation of histones H3 and H4 of promoter regions of defense-related genes occur in primed plants (Jaskiewicz et al., 2011). Epigenetic changes also occur during the transgenerational primed-state (Luna et al., 2012).

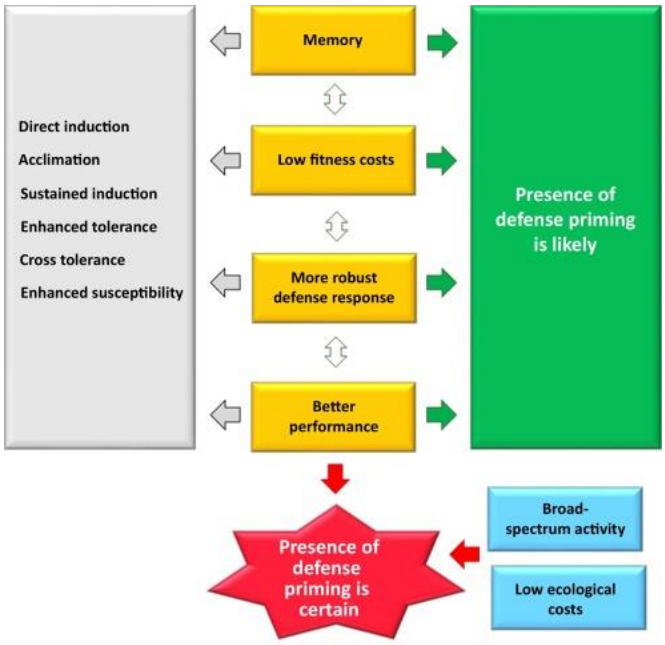

Fig. 1-3: How to recognize defense priming? Yellow squares represent the main criteria to find defense priming. More criteria are present (green arrows), likely is the presence of priming. If they are not accomplished, plants deploy other defense strategies but not priming (grey arrows). If the main criteria, a broad spectrum of activity and low ecological costs are present, priming is certain. Image from Martinez-Medina et al., 2016.

During the post-challenge primed state, primed plants show an enhanced perception of the attacker, increasing for example their responsiveness to PAMPs (Mauch-Mani et al., 2017). During PTI, ROS generation increases in primed plants, and an accelerated stomatal closure has also been observed. Important markers of the post-challenge priming state are also an enhanced callose deposition and an earlier stronger expression of genes involved in defense (Baccelli and Mauch-Mani, 2016; Mauch-Mani et al., 2017).

Among the best priming stimuli, the non-proteinogenic amino acid β-aminobutyric acid (BABA) has been known for 50 years for its resistance-inducing- capacity. Later, it turned out that it was also an ideal priming agent, which is effective against a wide variety of stresses, both biotic and abiotic, and its presence has been described in plants (Cohen et al., 2016; Thevenet et al., 2017).

1.2. The multifaceted roles of BABA

1.2.1. The discovery of BABA-induced resistance

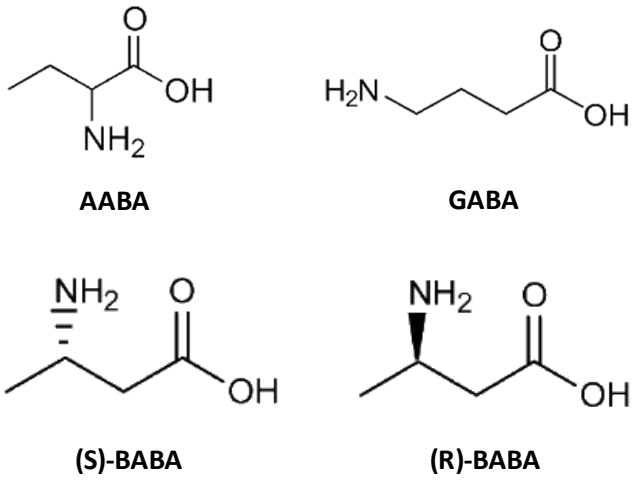

Fig. 1-4: Isomers of the aminobutyric acid and enantiomers of β-aminobutyric acid.

BABA is one of the three isomers of the aminobutyric acid, along with α-aminobutyric acid (AABA) and γ-aminobutyric acid (GABA). Two enantiomers of BABA exist, (R)-BABA and (S)-BABA (**Fig. 1-4**). Papavizas (1964) discovered that the soil-drench application of BABA controlled the spread of the root pea pathogen *Aphanomyces euteiches*. BABA was already effective if applied ten days before pathogen inoculation, although BABA application was more effective three days before *A. euteiches* inoculum. Papavizas (1964) argued that BABA prevented the appearance of disease symptoms instead of acting as an antimicrobial compound. Three decades later, Cohen et al. (1994) tested the efficacy of BABA, AABA, and GABA in tomato against late blight. Interestingly, only BABA was able to induce a considerable resistance against *Phytophthora infestans* (**Fig. 1-5**). Moreover, BABA did not block sporangial germination *in vitro* nor cytospore germination on tomato leaves, confirming the BABA-induction of the plant immune system argued by Papavizas (1964). Furthermore, BABA was able to increase the concentrations of PR proteins in infected and non-infected plants, and this induction was stronger compared to AABA and GABA (Cohen et al., 1994). Subsequently, Cohen et al. (1994) tested the efficacy of BABA in tobacco plants infected with *Peronospora tabacina*, the causal agent of blue mold. As observed in tomato, BABA was able to induce resistance in tobacco and to boost *PRs* transcripts concentrations (Cohen et al., 1994). Furthermore, Cohen et al. (1994) discovered the stereospecific activity of BABA. In fact,

applying the two enantiomers of BABA, only (R)-BABA induced resistance against *P. tabacina*, while (S)-BABA was completely ineffective (Cohen et al., 1994).

BABA-IR was then documented in several other pathosystems. BABA was effective against other oomycetes species and fungal pathogens as well. BABA application induces resistance against nematodes and insects, and viral and bacterial diseases as well (reviewed in Cohen et al., 2016) (**Table 1**).

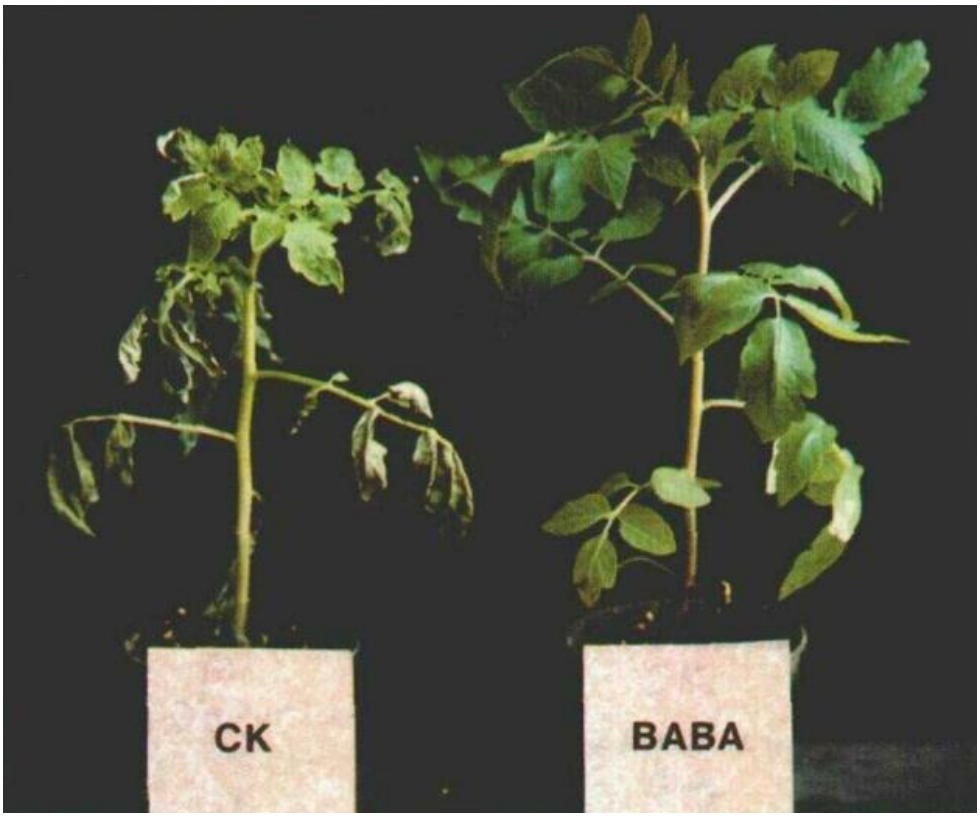

Fig. 1-5: Effects of BABA on tomato plants against late blight. Control plants (CK) were sprayed with water, while treated plants (BABA) with a solution of 2000 µg/mL of BABA. The image is taken from Cohen et al. (1994).

Class	Pathogen	Host	Response	References
Oomycetes	*Aphanomyces euteiches*	Pea	Reduction of pathogen growth	Papavizas (1964)
	Phytophtora infestans	Tomato	Reduction of pathogen spread, Increased PR proteins induction	Cohen et al. (1994)
		Potato	Increased β-1,3-glucanase and aspartyl protease concentrations, *PR* genes up-regulation, callose papillae, phenocopy HR, increased phytoalexins and phenols, block of pathogen growth	Altamiranda et al. (2008) Si-Ammour et al. (2003) Bengtsson et al. (2014)
	Peronospora tabacina	Tobacco	Increased *PR* transcripts, block of pathogen growth	Cohen (1994)
	Pythium aphanidermatum	Ginger	Increase in antimicrobial proteins concentrations, block of pathogen growth	Karmakar et al. (2003)
	Plasmopara viticola	Grapevine	Callose apposition and lignification of the cell wall, block pf pathogen growth and sporulation	Hamiduzzaman et al. (2005)
	Bremia lettucae	Lettuce	*PR* genes up-regulation, callose papillae, phenocopy HR, increased phytoalexins and phenols, block pf pathogen growth and sporulation	Cohen et al. (2010)
	Hyaloperonospora arabidopsidis	Arabidopsis	*PR* genes up-regulation, callose papillae, phenocopy HR, trail necrosis, block pf pathogen growth, and sporulation	Zimmerli et al. (2000) Ton et al. (2005)
Ascomycetes	*Botrytis cinerea*	Strawberry	ROS production and boosting chitinase, phenyl ammonia-lyase, and β-1,3-glucanase activity. Induced *PR1* gene. Control of pathogen growth	Wang et al. (2016)
		Tomato	Reduction of pathogen growth in fruits	Luna et al. (2016) Wilkinson et al. (2018)
		Arabidopsis	Control of pathogen growth, induction of *PR1* gene	Zimmerli et al. (2001)
	Alternaria brassicicola	Arabidopsis	Control of pathogen growth, induction of *PR1* gene, callose accumulation in the cell wall	Ton and Mauch-Mani (2004) Flors et al. (2008)

	Plectosphaerella cucumerina	Arabidopsis	Control of pathogen growth, induction of *PR1* gene, callose accumulation in the cell wall.	Ton and Mauch-Mani (2004)
Zygomycota	*Aspergillus flavus*	Pistachio	Reduction of fungal growth and fungal aflatoxin accumulation in kernels. Higher phenol and flavonoid production and increased PAL activity.	Aghdam et al. (2020)
Deuteromycota	*Rhizopus stolonifer*	Peach	Induction of *PR-like* genes, enhanced lignification, increased chitinase, and β-1,3-glucanase enzymatic activity.	Wang et al. (2018)
Basidiomycota	*Puccinia triticina*	Wheat	Control of pathogen growth, callose deposition at the site of pathogen penetration, ROS burst, phenocopy HR	Bellameche et al. (2020)
Aphids	*Acyrthosporium pisum*	*Vicia fava*	Control of pest spread	Hodge et al. (2005)
	Acyrthosporium pisum	*Medicago truncatula*	Control of pest spread	Hodge and Powell (2012)
	Brevicoryne brassicae	Different Brassicae	Control of pest spread	Hodge et al. (2006)
	Myzus persicae	Different Brassicae	Control of pest spread	Hodge et al. (2006)
Lepdoptera	*Trichoplusia ni*	Different Brassicae	Control of pest spread	Hodge et al. (2006)
	Plutella xylostella	Different Brassicae	Control of pest spread	Hodge et al. (2006)
Nematodes	*Meloidogyne graminicola*	Rice	Reduction of juvenile penetration in roots, reduce nematode development, increased callose deposition, and lignification, ROS burst	Ji et al. (2015)
Viruses	TMV	Tobacco	Reduction of the number of viral-derived lesions	Siegrist et al. (2000)
	GLRaV-1	Grapevine	Decreased viral transcripts gradually from older leaves to younger shoots	Csikász-Krizsics et al. (2013)

Bacteria	*Ralstonia solanacearum*	Tomato	Increased polyphenol oxidase activity, increased phenols concentration, ROS burst	Hassan and Abo-Elyousr (2013) Barilli et al. (2010) Vanitha et al. (2009)
	Clavibacter michiganensis	Tomato	Increased peroxidase activity, ROS burst	Baysal et al. (2005)
	Pseudomonas syringae	Arabidopsis	Up-regulation of *PR-1* gene, control of pathogen growth	Zimmerli et al. (2000)

Table 1-1: BABA induction of resistance against pathogens in different plant species.

1.2.2. BABA-induced resistance during biotic stress

1.2.2.1. BABA-IR against oomycetes

BABA-IR has been assessed in the *P. infestans*/potato pathosystem (Cohen, 2000; Si-Ammour et al., 2003; Altamiranda et al., 2008). Pre-treatment with foliar application of BABA confers resistance to harvested tubers of both Shepody susceptible and Kennebec moderate resistant potato cultivars inoculated with a *P. infestans* zoospores suspension. Indeed, tubers of BABA-treated potatoes express high concentrations of phytoalexins and phenols in response to pathogen attack (Andreu et al., 2006). Furthermore, BABA-treated Shepody tubers showed a two-fold increase in β-1,3-glucanase and aspartyl protease after inoculation. At the same time, in the resistant variety, the induction was six-fold higher than in mock-treated plants (Andreu et al., 2006). Foliar application of 10 mM BABA confers resistance also to both Bintje susceptible and Ovatio partially resistant varieties (Bengtsson et al., 2014). BABA-IR was associated with the apparition of microscopic HR-like lesions. Furthermore, papillae occurred around HR-like microlesions and at the site of pathogen penetration (**Fig. 1-6**) (Bengtsson et al., 2014). Finally, *PR-1* expression was boosted in both susceptible and partially resistant varieties, although Ovatio cultivar showed a stronger induction of *PR-1* gene (Bengtsson et al., 2014).

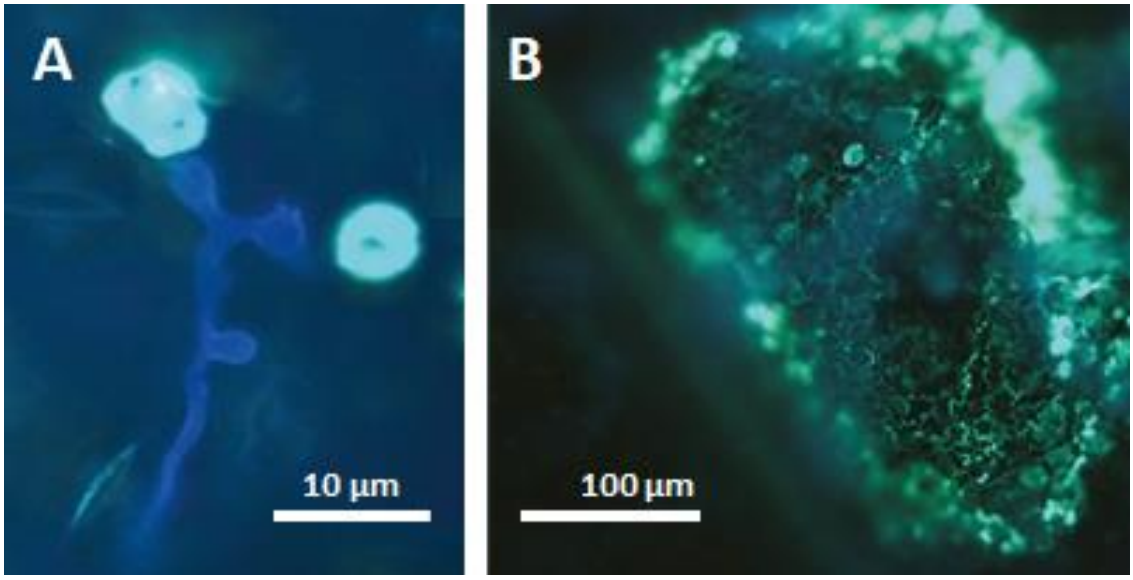

Fig. 1-6: Enhanced callose deposition and hypersensitive response in BABA-treated potatoes against late blight. A: callose papillae stained with aniline blue visible around the *P. infestans* appressorium, stained with Utivex 2B. **B**: HR-like lesions and callose deposits around the necrotic lesions. Image taken from Bengtsson et al., 2014.

BABA-IR is also active against *Pythium aphanidermatum* in ginger and several species of *Perenosporacae*, such as *Plasmopara viticola* in grapevine, *Bremia lettucae* in lettuce, and different *Hpa* isolates in Arabidopsis (Cohen et al., 1999; Zimmerli et al., 2000; Karmakar et al., 2003; Ton et al., 2005; Cohen et al., 2007). Zimmerli et al. (2000) elucidated the mechanisms of BABA-IR against oomycetes in Arabidopsis. Susceptible Arabidopsis Col-0 plants treated with a BABA solution were able to block the virulent *Hpa* isolate NOCO. The acquired resistance was associated with the apposition of callose papillae at the site of pathogen penetration, similarly as occurring in potato against late blight (Zimmerli et al., 2000). By contrast, at lower concentrations, BABA boosted a phenocopy HR at the site of pathogen attack. Moreover, in the case of a pathogen growth between mesophyll cells, BABA activated trailing necrosis around the developing hyphae (Zimmerli et al., 2000).

Similar results were obtained in detached cotyledons of BABA-treated lettuce inoculated with *Bremia lettucae* (Cohen et al., 2010). Indeed, pathogen mycelium was not detected in BABA-treated plants, while a massive callose deposition was present at the site of pathogen penetration. Furthermore, epidermal cells became lignified once pathogen attempted to penetrate (Cohen et al., 2010). Callose accumulation and lignification were also found in leaves of BABA-treated grapevine infected with *Plasmopara viticola*. Callose accumulation was furthermore stronger around the site of *P. viticola* sporangiophore extrusion and in guard cells (Hamiduzzaman et al., 2005). However, as occurring in potato, the strength of BABA-IR

against downy mildew in grapevine varies with the degree of resistance present in the host. In the resistant cultivar Solaris no sporulation could be detected, while in the susceptible grape cultivar Chasselas few sporangiophores could extrude, although weak and distorted (Hamiduzzaman et al., 2005).

1.2.2.2. BABA-IR against Fungi

BABA-IR occurs against different ascomycete species (Zimmerli et al., 2001; Ton and Mauch-Mani, 2004; Cohen et al., 2016). As an example, in *Botrytis cinerea*-inoculated strawberry fruits, BABA controlled pathogen spread, prompting ROS production, and boosting chitinase, phenyl ammonia-lyase, and β-1,3-glucanase activities (Wang et al., 2016). A protective effect of BABA against grey mold was also observed in tomato. Tomato plants treated with BABA at the seedling stage showed resistance to *B. cinerea* not only in adult leaves but further in mature fruits (Luna et al., 2016; Wilkinson et al., 2018). Induced resistance against *B. cinerea* was also assessed in tomato adult plants after incubating seeds for seven days with a BABA solution (Luna et al., 2016). BABA-treated *B. cinerea*-inoculated strawberries respond to the infection, increasing transcription of the *PR-1* gene. The same results were obtained in Arabidopsis Col-0 were lesion diameter was reduced (**Fig. 1-7**), and *PR-1* transcripts increased (Wang et al., 2016; Zimmerli et al., 2001). BABA-IR against the necrotrophic ascomycetes *A. brassicicola* and *P. cucumerina* also increase *PR-1* transcript levels (Ton and Mauch-Mani, 2004). However, differently as occurring against grey mold, BABA response to *P. cucumerina* and *A. brassicicola* is based on an early callose deposition at the site of pathogen penetration (Ton and Mauch-Mani, 2004). Indeed, the treatment of Arabidopsis plants with BABA and with the callose inhibitor 2-deoxy-D-glucose caused the lost of the BABA-induced resistance capacity (Ton and Mauch-Mani, 2004).

BABA application also induced resistance against deuteromycota and zygomycota (Wang et al., 2018; Aghdam et al., 2020). BABA application, for example, induced resistance against *Aspergillus flavus* in harvested pistachio fruits, reducing the fungal aflatoxin accumulation in kernels as well. Furthermore, BABA application boosted higher phenol and flavonoid production in pistachio kernels, and also an increased PAL enzymatic activity (Aghdam et al., 2020). BABA was effective against the zygomycete *Rhizopus stolonifer* in peaches. BABA increased the induction of *PR-like* genes in peach fruits infected with *R. stolonifer*. Moreover, BABA enhanced lignification in peaches fruits in response to pathogen attack and chitinase and β-1,3-glucanase enzymatic activity as well (Wang et al., 2018).

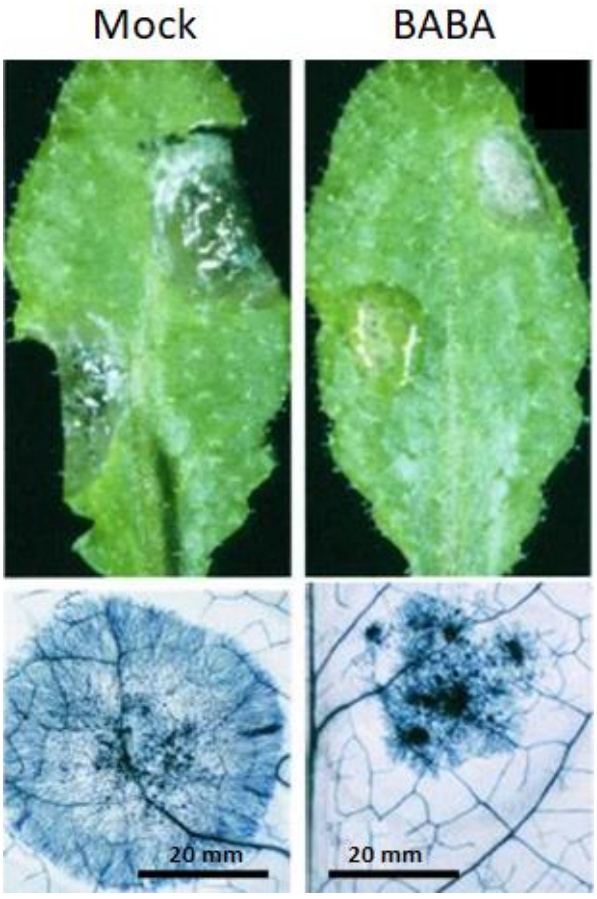

Fig. 1-7: BABA-induced resistance in Arabidopsis Col-0 against *Botritis cinerea*. Arabidopsis leaves after 3 days post drop inoculation with a suspension of *B. cinerea* conidia. Mock-treated plants (left side) show larger lesions compared to soil drench BABA-treated plants (right side). Micrographs show lactophenol-trypan blue-stained leaves. Image taken from Zimmerli et al. (2001).

BABA is an efficient molecule in the control of biotrophic basidiomycetes (Amzalek and Cohen, 2007; Cohen et al., 2016; Bellameche et al., 2020). In *Puccinia triticina*-infected wheat plants, for example, soil-drenched BABA diminished the formation of uredia and chlorotic flecks in a concentration-dependent manner (Bellameche et al., 2020). Furthermore, BABA blocked sub-stomatal vesicles and haustoria formation. However, urediospores can germinate and form appressoria, confirming the non-toxicity effect of BABA on pathogen germination (Bellameche et al., 2020). Moreover, BABA provoked the accumulation of callose at the infection site already 48 hours post-inoculation. Furthermore, DAB staining on leaves of BABA-treated wheat plants infected with rust showed a strong increase in H_2O_2 concentrations (Bellameche et al., 2020). Both ROS and callose were mainly detected in guard cells; however, BABA led to an accumulation of both chemical species in mesophyll cells. This BABA-mediated HR-like response in mesophyll prevented the formation of haustoria and blocked pathogen growth in leaves (Bellameche et al., 2020).

1.2.2.3. BABA-IR against insects and nematodes

BABA is effective in blocking nematodes and insects attacks in several plant species (Oka and Cohen, 2001; Hodge et al., 2005; Hodge et al., 2006; Hodge et al., 2011; Ji et al., 2015). Hodge et al. (2005) demonstrated that soil drenching a BABA solution, *Vicia faba* plants became resistant to the pea aphid *Acyrthosporium pisum*. Neither the ingestion of a BABA-sucrose solution by nymphs nor topical and residual tests on older *A. pisum* provoked toxic effects (Hodge et al., 2005). Larvae of the aphididae *Brevicoryne brassicae* and *Myzus persicae*, and of the Lepidoptera *Trichoplusiani* and *Plutella xylostella*, feeding on leaves of different BABA-treated Brassicae species, showed a decrease in average body weight compared to larvae feeding non-treated plants (Hodge et al., 2006). Moreover, treatment on *Vicia faba* and *Medicago truncatula* with soil-drench BABA led to the malformation of *A. pisum* nymphs, characterized by indistinguishable limbs and antennae and complete impaired survival rate (Hodge and Powell, 2012). Nevertheless, although effective in controlling insect attacks, BABA negatively affected multitrophic interactions. Hodge et al. (2011) demonstrated that BABA negatively impacted on the growth of the braconic aphid parasitoid *Aphidus ervi*. In *A. pisum*-attacked *Vicia faba* plants treated with a 50 μM BABA solution, the production of mummies and adults of *A. ervi* was reduced, with a stronger effect when BABA was applied prior to oviposition. This reduction in parasitoid weight and growth was correlated to the negative effect of BABA on the aphid-prey population (Hodge et al., 2011). Unfortunately, the cellular mechanisms controlling BABA-IR against insects are not known yet.

The cellular response of BABA-treated plants to nematodes has been deeply described (Wilkinson et al., 2018). A notable example comes from *Oriza sativa* cv. Nipponbare and cv. Taipei 309, where BABA treatment reduced the number of penetrating *Meloidogyne graminicola* juvenile individuals in plant roots (Ji et al., 2015). Furthermore, BABA not only impeded *M. graminicola* penetration but also blocked the development of nematodes from the juvenile second stage to more adult stages (Ji et al., 2015). BABA-treated rice plants blocked nematode penetration in roots, increasing callose deposition in galls. In fact, BABA-treated plants exhibited a strong up-regulation of the callose synthase gene *OsGSL3* and downregulation of the callose-hydrolise gene *OsGNS5* (Ji et al., 2015). During *M. graminicola* penetration, BABA upregulated genes involved in lignin biosynthesis, such as *OsPAL*, *OsC4H*, and *OsCAD6*. At the same time, in mock-treated inoculated plants a nematode-dependent downregulation of these genes occurred (Ji et al., 2015). Finally, in BABA-treated rice plants,

nematode penetration stimulated a more vigorous ROS burst compared to non-treated plants (Ji et al., 2015).

1.2.2.4. BABA-IR against viruses and bacteria

The first evidence of BABA- IR against viruses came from Siegrist et al. (2000), which documented the role of BABA in tobacco against Tobacco mosaic virus (TMV). In this work, both tobacco plants carrying the *Nicotiana glutinosa*-derived *N* gene, conferring hypersensitivity to TMV, and wild-type tobacco Xanthi-nc plants resulted resistent to TMV after treating with a 10 mM BABA solution. At this concentration, BABA induced a reduction in the number of viral-derived lesions, while at lower concentrations, the activity of BABA-IR against virus decreased (Siegrist et al., 2000). Similar results were obtained in *Vitis vinifera* cv. Leányka infected with Grapevine leafroll-associated virus-1 (GLRaV-1) (Csikász-Krizsics et al., 2013). Indeed, GLRaV-1-inoculated plantlets, soil-drenched every week with a BABA solution, reported a high rate of cane development, while mock-treated plants died. Furthermore, viral transcripts decreased gradually from older leaves to younger shoots (Csikász-Krizsics et al., 2013).

In contrast to viruses, more research has been reported about BABA-IR against bacteria (Zimmerli et al., 2000; Jakab et al., 2001; Baysal et al., 2005; Hassan and Abo-Elyousr, 2013; Li et al., 2016). As occuring with other pathogens, BABA enhanced the host immune system once infected with bacteria. For example, BABA-treated tomato cv Super Jackal plants increased polyphenol oxidase activity after *Ralstonia solanacearum* isolate RS7 infection (Hassan and Abo-Elyousr, 2013). Polyphenol oxidase is a copper-containing enzyme that converts phenol compounds into quinines with antimicrobial activity, involved in the resistance against *R. solanaceraum* (Vanitha et al., 2009; Barilli et al., 2010). At the same time, BABA-soil drench treatment induced an increase of phenolic compounds in unchallenged and inoculated plants (Hassan and Abo-Elyousr, 2013). Furthermore, hydrogen peroxide production increased after BABA treatment, while catalase activity decreased concomitantly (Hassan and Abo-Elyousr, 2013). Catalase is an antioxidant enzyme catalyzing the conversion of hydrogen peroxide into water and oxygen, reducing thus ROS burst (Bhattacharjee, 2005). Interestingly, BABA-sprayed application increased POX activity in tomato seedlings cv. Rio Grande already three days post-inoculation with *Clavibacter michiganensis* spp *michiganensis* (*Cmm*), the causal agent of bacterial canker of tomato (Baysal et al., 2005; Nandi et al., 2018). Therefore, BABA-IR against *Cmm* in tomato increased the concentration of hydrogen peroxide three days

after inoculum, although BABA induced H_2O_2 concentration in non-inoculated plants as well (Baysal et al., 2005). BABA also induces resistance against different species of the *Pseudomonas* genera (Cohen et al., 2016). Zimmerli et al. (2000), for example, demonstrated that BABA is effective against the strain *DC 3000* of *Pseudomonas syringae pv tomato* (*Pst* DC3000), decreasing symptoms and increasing *PR-1* gene expression (Siegrist et al., 2000).

1.2.3. Hormonal signaling in BABA-induced resistance

Pst DC3000 is a pathogenic bacterium infecting both tomato and Arabidopsis, considered one of the better model organisms to study the hormonal signaling in plant-pathogen interaction (Xin and He, 2013). For this reason, Zimmerli et al. (2000) examined BABA-IR in the *npr1-1* mutant and in the transgenic Arabidopsis plants harboring a salicylate hydroxylase gene *NahG*. *NahG* is a *Pseudomonas putida* gene converting SA into catechol (Lawton et al., 1995). Interestingly, BABA-IR showed no reduction of bacterial titer in both *NahG* and *npr1-1*. Mutants in ET- and JA-signaling *jar1* and *etr1* displayed the BABA-IR. These data demonstrated the requirement of SA in BABA-IR against bacteria (**Fig. 1-8**). Siegrist et al. (2000) demonstrated that BABA failed to induce resistance against TMV in the Tobacco *NahG* plants (Gaffney et al., 1993). Later, other works showed that a functional SA pathway is required for BABA-IR in potatoes infected with *P. infestans* and in Arabidopsis versus *B. cinerea* as well. Indeed, *PR-1* gene induction in BABA-treated Arabidopsis with *B. cinerea* was altered in *NahG* and *npr1-1* mutants (Zimmerli et al., 2001; Eschen-Lippold et al., 2010). Furthermore, Pastor et al. (2014) demonstrated that soil-drenched BABA solution boosted SA and SA-glycosides concentrations in Arabidopsis Col-0 seedlings. Interestingly, BABA-sprayed tobacco leaves expressed higher SA concentrations compared to mock-treated plants (Siegrist et al., 2000). In BABA-treated tomato plants, total SA, and free SA are accumulated after infection with *Ralstonia spolanacearum* (Hassan and Abo-Elyousr, 2013). BABA also potentiates the expression of JA-regulated genes such as *LOX-9* and *PR-4* in grapevines infected with *P. viticola* (Hamiduzzaman et al., 2005). Furthermore, Pastor et al. (2014) demonstrated that BABA perception in Arabidopsis stimulates the synthesis of OPDA and JA-Valine conjugate. Interestingly, in BABA-treated *npr1-1* and *NahG* infected with *B. cinerea* the expression of *PDF1.2* increased compared to the wild type. By contrast, *PR-1* transcript levels increased in *etr1-1* and *coi1-1* mutants treated with BABA. This data suggest that when either the JA/ET or the SA dependent signaling is disrupted, BABA potentiates the other to set up induced- resistance (Zimmerli et al., 2001).

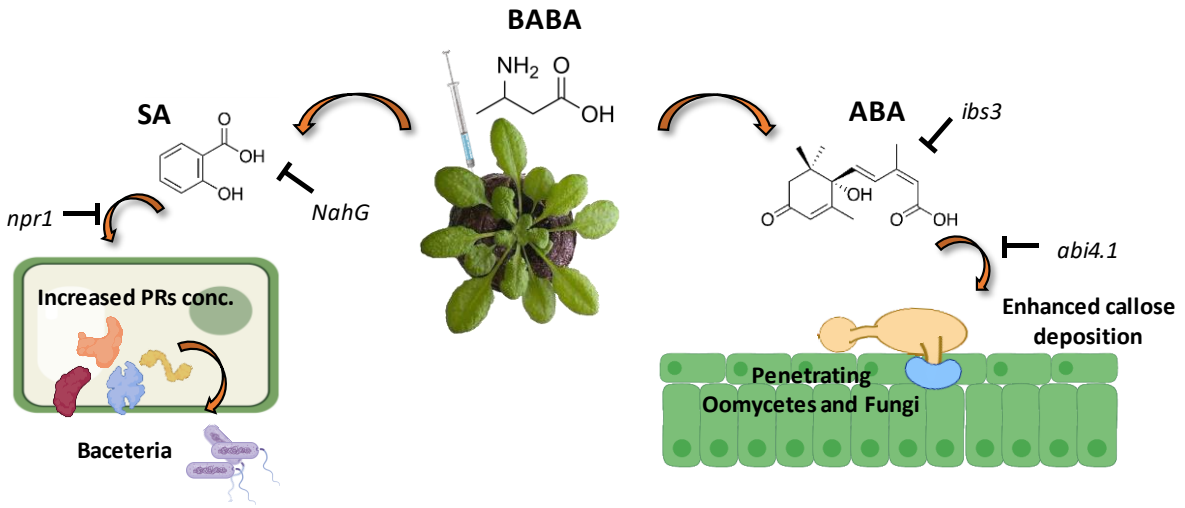

Fig. 1-8: Hormonal pathways involved in BABA-induced resistance. Soil-drench BABA treatment to Arabidopsis confers resistance towards bacteria, activating SA-signaling, and consequently increasing PR proteins concentrations. NahG and *npr1* result impaired in SA-dependent BABA-IR. The response to penetrating pathogens activates the ABA-dependent callose deposition. The biosynthetic mutant *ibs3* and the insensitive mutant *abi4.1* resulted impaired in ABA-dependent enhanced callose deposition.

When infected with the oomycete *Hpa* and the necrotrophic fungus *P. cucumerina*, both *NahG* and *npr1-1* showed an unaffected BABA-IR (Zimmerli et al., 2000; Ton and Mauch-Mani, 2004; Van der Ent et al., 2009). Moreover, BABA was unable to protect the ABA-signaling mutant *abi4-1* and the ABA-biosynthetic mutant *aba1-5* against infection with *P. cucumerina*. Similar results were obtained with the ABA-deficient mutant *npq2-1* against *Alternaria brassicicola*. These data revealed the existence of an additional ABA-dependent defense mechanism during BABA-IR (**Fig. 1-8**) (Ton and Mauch-Mani, 2004; Flors et al., 2008). This ABA-dependent BABA-IR boosts an enhanced accumulation of callose papillae at the site of pathogen penetration. Indeed, the callose synthase mutant *pmr4-1* during *A. brassicicola* infection and *abi4-1* inoculated with *P. cucumerina* both failed in BABA-primed callose deposition, but not in basal callose deposition (Ton and Mauch-Mani, 2004; Flors et al., 2008). At the same time, repeated treatments with high doses of BABA led to the formation of large callose deposits at the ovule micropyle (Jakab et al., 2001; Kocsis and Jakab, 2008). Starting from these observations, Ton et al. (2005) identified three Arabidopsis mutants impaired in BABA-induced sterility (*ibs*). The first, *ibs1*, possesses a mutation in a gene encoding a cyclin-dependent kinase-like protein, which regulates SA-dependent and transgenerational BABA-priming (Slaughter et al., 2012). *IBS2* is expressed in immature pollen and siliques, and the mutant allele *ibs2* affects a phosphoinositide phosphatase regulating phosphoinositide concentrations (Despres et al., 2003). Finally, *ibs3*, mutated in the gene *ABA1/NPQ2*, encodes for a zeaxanthin epoxidase implied in the synthesis of ABA (Koornneef

et al., 1982; Zhu, 2002). *ibs2* and *ibs3* failed to express BABA-enhanced callose deposition after *Hpa* strain EMWA infection (**Fig. 1-8**). The two genes were also impaired in basal and BABA-induced tolerance for salt stress and failed to express the ABA-activated genes *RAB18* and *RD29A* under osmotic stress in both BABA-primed and mock-treated plants (Ton et al., 2005). Effectively, BABA-primed salt-treated Arabidopsis accumulated more ABA compared to wild-type, while *abi4-1* and *aba1-5* failed to express BABA-induced tolerance to salt stress (Jakab et al., 2005) .

1.2.4. BABA-induced tolerance to abiotic stress

Jakab et al. (2005) were the first to test the acclimation capacities of BABA against osmotic stress. In this work, Arabidopsis Col-0 plants were therefore soil-drenched with a 300 µM BABA solution one day before stopping watering. Interestingly, after one week from water depletion, there were less wilted plants following BABA treatment compared to water treatment. Similar results took place in BABA-treated soil-drenched with 300 mM NaCl solution, where the wilting rate was reduced by 50% (Jakab et al., 2005). At the same time, AABA and GABA-treated plants showed symptoms comparable to mock-treated plants. Interestingly, BABA-pretreated Arabidopsis transferred from a high-humidity environment to low humidity conditions showed reduced stomatal conductance compared to water-treated plants. Nevertheless, in salt-challenged plants, although proline levels increased in both treatments, BABA did not enhance this response, and concentrations remain similar to mock-treated plants (Jakab et al., 2005).

BABA-treatment on Arabidopsis also causes cadmium tolerance, alleviating, for example, Cd-dependent root growth inhibition (Cao et al., 2009). This tolerance is associated with a primed increase in the GSH-biosynthetic gene *GSH1* transcript concentrations after Cd application on the soil. Furthermore, co-application of BABA and buthionine sulfoxime, an inhibitor of GSH synthesis, on Cd-treated Arabidopsis seedlings abolished the BABA-improvement of root growth (Cao et al., 2009). BABA also increases acquired thermo-tolerance but has no effects on basal thermotolerance. Moreover, BABA-induced thermotolerance involves the heat-shock factor HSP101 that plays a role in refolding proteins during heat stress. In fact, in the HSP101 mutant *hot1-1* BABA did not protect either in basal or in acquired thermotolerance (Zimmerli et al., 2008).

By the side, Ben Rejeb et al. (2018) looked at the priming activity of BABA in tomato after treatment with a combination of salt stress and *B. cinerea* infection. The authors found that

callose and lignin deposition against *B. cinerea* increased in BABA and NaCl co-treated tomato plants compared to naïve plants. Furthermore, the expression pattern of *PR1* and *PR5* in BABA-treated and double stressed tomato plants increased, and rapid accumulation of H_2O_2 also was observed (Ben Rejeb et al., 2018).

1.2.5. BABA as a priming agent

The broad spectrum of stress-resistance displayed and the different hormonal signaling boosted suggest a mechanism linking BABA to priming.

As seen in other pathosystems, BABA-IR against *Pst* DC3000 in Arabidopsis boosts the induction of PR proteins. Zimmerli et al. (2000) demonstrated that BABA-treated Arabidopsis Col-0 inoculated with the *Pst* DC3000 showed increased *PR-1* transcripts already at eight hours post-inoculation, earlier than in mock-treated plants (Zimmerli et al., 2000). This faster induction of *PR-1* transcript is similar to that provoked during the incompatible reaction between Arabidopsis Col-0 and the avirulent strain *Pst* AvrRpt2 (Zimmerli et al., 2000). Interestingly, BABA treatment in non-inoculated plants provoked only a weak induction only three days post-application (Zimmerli et al., 2000). This effect has been the first evidence of the BABA-induced priming in plants (Zimmerli et al., 2000; Conrath et al., 2002).

van Hulten et al. (2006) demonstrated that BABA works as defense inducer or as a priming agent, depending on the concentrations applied. At higher concentrations, BABA caused a strong reduction in plant growth and seed production, in the same order of magnitude of BTH, both in mock and in *Hpa* and *Pst* inoculated plants. Contrarily, concentrations between 10 and 25 mg/L of BABA induced high levels of protection against *Hpa* and *Pst* and low fitness costs. These data were comparable to those obtained in the constitutive priming *edr1-1* mutant. Furthermore, van Hulten et al. (2006) showed that *PR-1* gene expression in plants treated with low concentrations of BABA was not enhanced until pathogen inoculation.

During the priming phase, BABA induces the biosynthesis of primed compounds that will help the plant to deal with the next challenge. BABA enhances the accumulation of (L)-pipecolic acid, which seems to play a central role in mediating BABA-induced resistance to bacterial infection. Also, BABA increases cystein, methionine, glutamine, isoleucine, leucine, histidine, threonine, tyrosine, and phenylalanine accumulation (Návarová et al., 2012; Pastor et al., 2014). According to Pastor et al. (2014), BABA treatment of Arabidopsis stimulated the production of tricarboxylic acid cycle compounds, in particular malate, fumarate, isocitrate and

alpha-ketoglutarate. TCAs are utilized for the production of lipid derivatives, some of which are accumulated after BABA treatment, as octadecanoids, OPDA and JA, important in defense response. Moreover, SA acid and glucosides of SA are accumulated during the BABA priming phase.

Moreover, BABA increases the content of secondary metabolites, such as ferulic and caffeic acid (Pastor et al., 2014). Besides, different studies focused on the capacity of BABA to induce post-translational modifications. For example, the BABA-dependent enhanced responsiveness of PTI genes in Arabidopsis and common bean depend on the acetylation and demethylation of different residues of lysine in histone H3 (Po-Wen et al., 2013; Martínez-Aguilar et al., 2016). Long-lasting BABA priming lasts up to 4 weeks after treatment and requires the NPR1-regulatory system. Moreover, the histone methyltransferase KYP is required for long-lasting priming by BABA, based on the fact that the *kyp-6* mutant is impaired in long-lasting BABA-IR, which suggests a role in silencing suppressors genes of SA/NPR1- dependent genes (Luna et al., 2014a). Finally, Slaughter and colleagues (2012) demonstrated that the progeny of BABA-primed plants can inherit BABA-priming. Indeed, Arabidopsis plants derived from BABA-treated plants were resistant to *Pst* DC3000 and *Hpa*,and showed an accumulation of *PR* gene transcripts after challenge application. Furthermore, no BABA was detected in the next generation of primed plants, suggesting an inheritance of the priming state by post-transductional or epigenetic modifications (Slaughter et al., 2012).

1.2.6. BABA-receptor IBI1

Searching for novel signaling components in SA-independent BABA-mediated-immune response, Luna et al. (2014b) identified the *Impaired in BABA-induced Immunity 1* (*IBI1*) gene, encoding for an aspartyl-tRNA synthase (AspRS). The interaction between BABA and IBI1 is enantiomer specific. R-BABA application increased (L)-aspartate concentrations, blocking the canonical AspRS activity, while the S-BABA application did not replicate the same pattern.

Ibi1-1 controls SA-independent BABA priming. Indeed, BABA failed to induce resistance against *Hpa* and *P. cucumerina* in *ibi-1* mutants. This impaired resistance was associated with a lack in callose accumulation. However, ABA was able to induce resistance in *ibi1-1*, suggesting that IBI1 works upstream of ABA in BABA-IR. Furthermore, although the *ibi1-1* mutant was not impaired in SA-dependent immunity, it failed to express primed concentrations of *PR-1* transcripts after *Hpa* infection. This data demonstrates that IBI1 also controls the SA-

dependent BABA-induced priming. Finally, IBI1 is involved in the ISR signaling, as *P. fluorescens* WCS417r did not induce resistance against *Hpa* in *ibi1-1* (Luna et al., 2014b).

Confocal microscopy analysis on Arabidopsis transgenic lines 35S::IBI1-YFP revealed that IBI1 is mainly localized in the endoplasmic reticulum and in the cytoplasm regions surrounding ER. Treatment with BABA maintained the reticular localization of IBI1-YFP. *Hpa* infection provoked only a minor shift of the labeled IBI1 to the peripherical cytoplasm. However, BABA enhanced this response, as the labeled IBI1 translocated in the peripherical cytoplasm. Finally, chromatographic analysis of the immunoprecipitated fraction of (R)-BABA-treated IBI1-YFP transgenic plants confirmed the link between R-BABA and IBI1. Therefore, IBI1 can be considered *bona fide* the receptor of BABA in plants (Luna et al., 2014b).

Consequently, the question is: why would plants evolve a receptor for a xenobiotic compound? Among the various hypotheses considered, the endogenous presence of BABA in plant tissues was not suggested (Luna et al., 2014b).

1.3. Plant-produced BABA

1.3.1. Non-proteinogenic amino acids and first evidences of endogenous BABA in plants

Plants display a plethora of physical and chemical defenses to protect themselves by dangerous environments. Many non-protein amino acids have deterrent and toxic properties, although some of them, such as homoserine and ornithine, also participate in primary metabolism (Bell, 2003; Vranova et al., 2011; Yan et al., 2015). A well-characterized NPAAs is GABA, which has been showed to be involved in multiple physiological activities (Bown and Shelp, 2016).

Plants synthesize GABA through the decarboxylation of glutamate catalyzed by glutamate decarboxylase (GAD). The inhibition of GABA-transaminase (GABA-T), which produces succinic semialdehyde starting from GABA, led to an increase in GABA concentration (Shelp et al., 2012). Plants accumulate GABA during different stresses, both biotic and abiotic (Bown and Shelp, 2016). For example, the Arabidopsis *gad1 gad2* double mutant does not synthesize nor accumulate GABA after *Spodoptera litoralis* infestation (Scholz et al., 2015). Furthermore,

gad1 gad2 results affected in vegetative growth and maintains stomata opened under osmotic stress, causing thus oversensitivity to draught (Mekonnen et al., 2016). GABA regulates osmotic stress response by activating an H^+-ATPase to maintain membrane potential and enhancing Na^+-efflux from cytosol and intake into the vacuole (Su et al., 2019). Moreover, plants utilize GABA as a signaling molecule. Indeed, pollen tube guidance is mediated by a GABA gradient in female reproductive tissues. Still, GABA also plays a role in pollen tube growth, considering that GABA-T mutant *pop2-1*, which over-accumulates GABA, shows reduced pollen tube elongation (Palanivelu et al., 2003; Renault et al., 2011). GABA is transported into plant cells by the transporter AtGAT1 (Meyer et al., 2006). By contrast, the ALMT transporter proteins mediate the efflux of GABA with malate from the cytosol (Ramesh et al., 2018). Furthermore, plant ALMTs interact with GABA through a specific GABA-interacting motif, similar to that of human GABA receptors (Ramesh et al., 2015; Long et al., 2020).

β-amino acids are rare compared to proteinogenic α-(L)-aminoacids. Still, they represent important components of bioactive natural products, such as antibiotics or secondary metabolites, and could also be incorporated into proteins (Kudo et al., 2014). Plants synthesize β-amino acids utilizing α-amino acids precursors in reactions catalyzed by aminomutases. One example comes from the tyrosine aminomutase TAM1 that enters in the biosynthesis of (R)-β-tyrosine in rice (Feng et al., 2011; Yan et al., 2015).

The presence of BABA in bacteria has been described in the lysine fermentation pathway, where it is bound to the active group CoA and synthetized cleaving 3-keto-5-aminohexanoate (Kreimeyer et al., 2007). Furthermore, in *Streptomyces,* BABA is present as a precursor of β-glutamic-acid in the biosynthesis of the antibiotic incednine (Takaishi et al., 2012). Even if it was believed that plants do not synthesize BABA, a presence was reported in tomato root exudates, in *Eucalyptus regnans* and wine. However, the methods employed in these experiments had not been validated for isomer selectivity, or the higher concentrations detected represent GABA instead of BABA (Gamliel and Katan, 1992; Barrado et al., 2009; Pfautsch et al., 2009).

1.3.2. BABA as endogenous metabolite in plants

In 2017, Thevenet and colleagues changed the perspectives on BABA in plants. Firstly, they set-up an analytical method to extract, separate, and analyze the three isomers of aminobutyric acid in plant tissues. This method consists of an SPE purification technique to clean plant extracts and to concentrate BABA and the other isomers and a hydrophilic interaction liquid chromatography-tandem mass spectrometry (HILIC-MS/MS) for the separation and the detection of the three isomers. Furthermore, deuterium labeled-internal standards are added for the compensation of the analytical variability due to the extraction, instruments or matrix effects (**Fig. 1-9.A**). Afterward, they used this efficient method to analyze the concentrations of the three isomers in non-stressed Arabidopsis Col-0 leaves. Their data clearly showed that plants synthesize BABA, although at lower concentrations than GABA and AABA, respectively (**Fig. 1-9.A**).

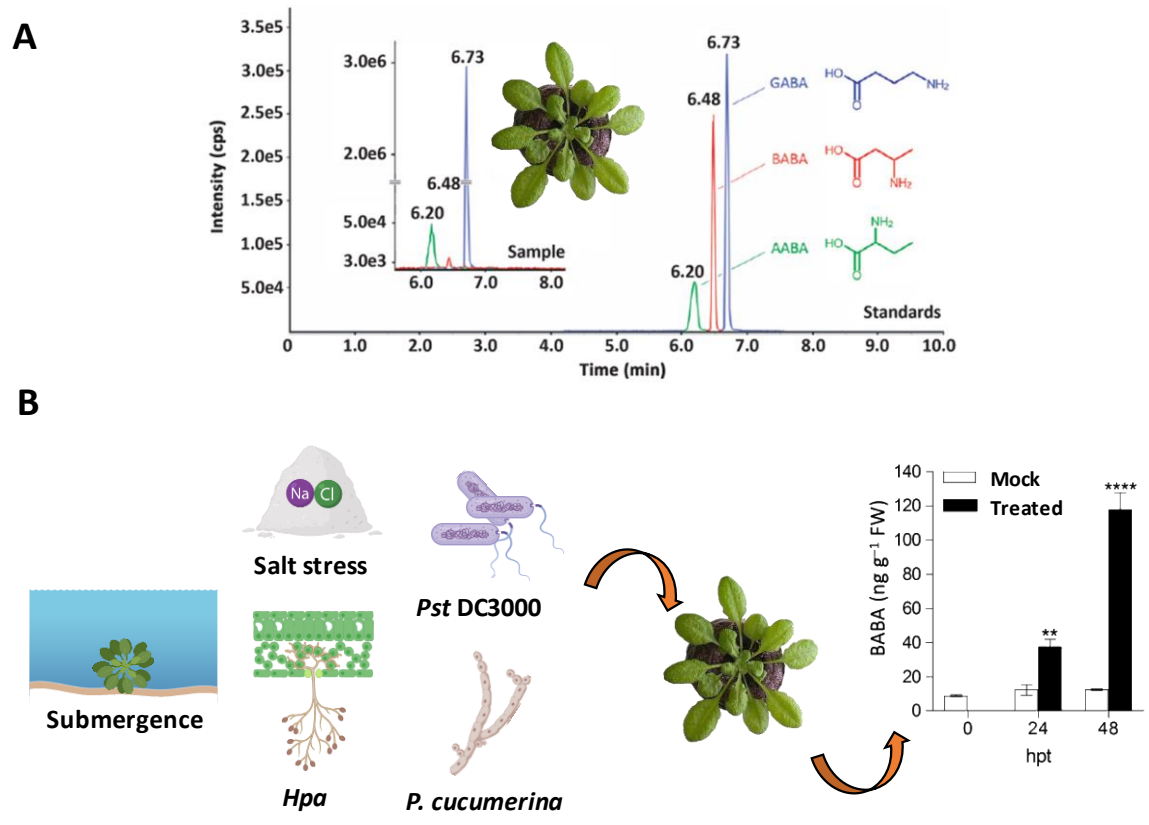

Fig. 1-9: BABA as plant metabolite. A: LC-MS chromatograms of Arabidopsis samples (smaller chromatogram) and standards (larger chromatograms). **B:** abiotic and abiotic stresses demonstrated to induce BABA in Arabidopsis. Image modified from Thevenet et al. (2017).

Afterward, the effects of both abiotic and biotic stress on endogenous BABA induction were validated (**Fig. 1-9.B**). After *P. cucumerina* inoculation, BABA started to accumulate 48 hours post-treatment (hpt), and after 72 hpt BABA levels increased up to five-fold. By contrast, a slight increase was reported after *Hpa* infection. Salt stress (200 mM NaCl) increased up to ten-fold the concentrations of BABA in Arabidopsis leaves 48 hpt, while in submergence, BABA levels rised to seven-fold compared to control plants. To test for feedback loop phenomena, the BABA content in *ibi1-1* mutant after salt stress was analyzed. No significant differences between *ibi1-1* and control Col-0 plants have been observed.

Finally, it was demonstrated that BABA is not only specific of Arabidopsis Col-0 but is widely present in the plant kingdom. Indeed, other Arabidopsis ecotypes were analyzed along with *Brassica rapa*, *Zea mays* cv. Delprim, teosinte, wheat, and the moss *Physcomitrella patens*, including by this way monocots, dicots, and a bryophyte. The detection of BABA in the moss *P. patens*, a model plant and a key transition point between green algae and higher plants, suggests a wide presence of this molecule in planta and a role in their basal metabolism.

1.3.3. The plant immune system regulates BABA concentrations in plants during biotic stress

Baccelli et al. (2017) demonstrated that the plant immune system regulates the accumulation of BABA after pathogen attack. Treatment with flg22 and Atpep2 of Col-0 plants led to an increased BABA accumulation (**Fig. 1-10**). Furthermore, Arabidopsis Ws-0 plants, which do not perceive flg22 due to an unfunctional FLS2 receptor, did not show BABA accumulation after flg22 application (**Fig. 1-10**). Similar results were obtained infiltrating Col-0 leaves with the DAMP AtPep2, although the induction capacity was less strong compared with flg22 treatment. These data clearly showed that plants accumulate BABA during PTI (Baccelli et al., 2017).

Moreover, infecting Arabidopsis Col-0 plants with *Pst* AvrRpt2, BABA levels raised earlier than following treatment with the virulent strain *Pst* DC3000. Thus, the earlier induction of BABA can be explained by the boosting of ETI (Baccelli et al., 2017). However, in plants inoculated with *Pst* AvrRpt2, BABA levels increased only in local infected tissues, while distal leaves showed no accumulation of BABA (Balmer et al., 2019).

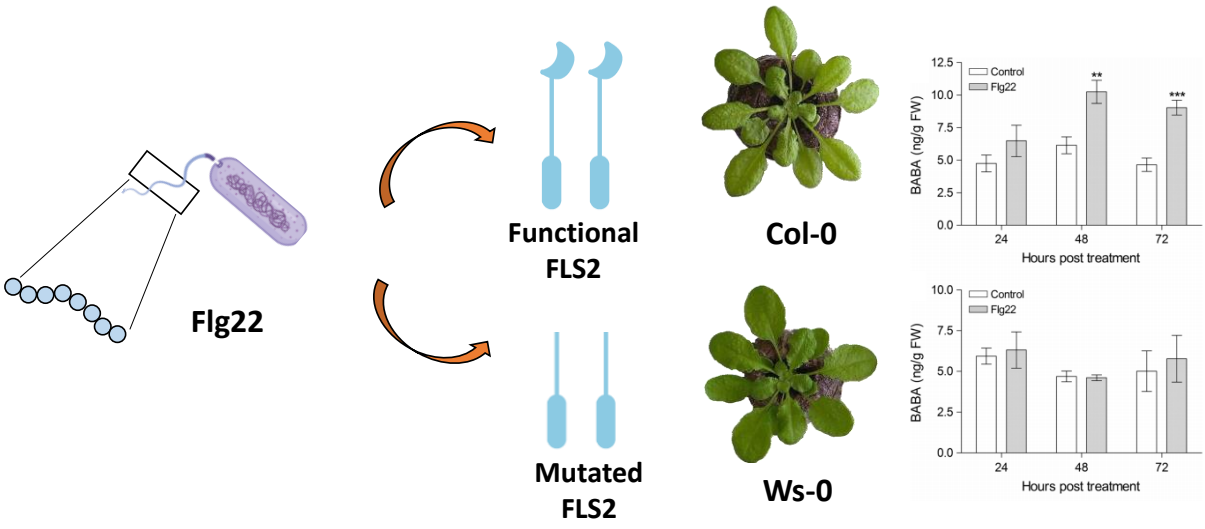

Fig. 1-10: Flg22 treatment leads to the accumulation of BABA in Arabidopsis. Col-0 accession accumulates BABA 48- and 72-hours post leaf infiltration with flg22. In Ws-0 plants BABA concentrations do not change after flg22 treatment. Image modified from Baccelli et al. (2017).

1.3.4. Spatial and temporal accumulation of BABA

The work of Balmer et al. (2019), improved the knowledge on endogenous BABA looking at BABA concentrations in different tissues and ages of plants. Indeed, considering the spatial distribution of BABA, authors showed that pollinated and un-pollinated flowers, siliques, and seeds were the site of maximal concentrations. Leaves, roots, and cauline leaves were instead the site of lowest accumulation (**Fig. 1-11.A**). Arabidopsis Col-0 plants gradually increased BABA concentrations after germination, reaching a peak at nine weeks post-germination, when the symptoms of senescence appear (**Fig. 1-11.A**). Detached leaves exposed to continuous dark conditions expressed induced senescence. BABA also accumulated in induced-senescent leaves at 2- and 7-days post-detachment. Authors suggested that this age-related BABA accumulation may be explained by the involvement of BABA in ARR (Balmer et al., 2019).

Finally, Balmer et al. (2019) discovered that the *constitutive expressor of PR gene 5-2* (*cpr5-2*) Arabidopsis mutant expressed higher levels of constitutive BABA compared to Col-0 wild type. Furthermore, concentrations of BABA increased with age in *cpr5-2* (**Fig. 1-11.B**). *Cpr5-2* shows constitutive defense and accelerated senescence phenotype, and additionally, an elevated ROS burst (Bowling et al., 1997; Clarke et al., 2000; Jing et al., 2007; Yoshida et al., 2002). This elevated ROS concentrations may explain the elevated BABA levels in *cpr5-2*. However, when treated with H_2O_2, BABA concentrations were similar in treated and untreated plants (Balmer et al., 2019).

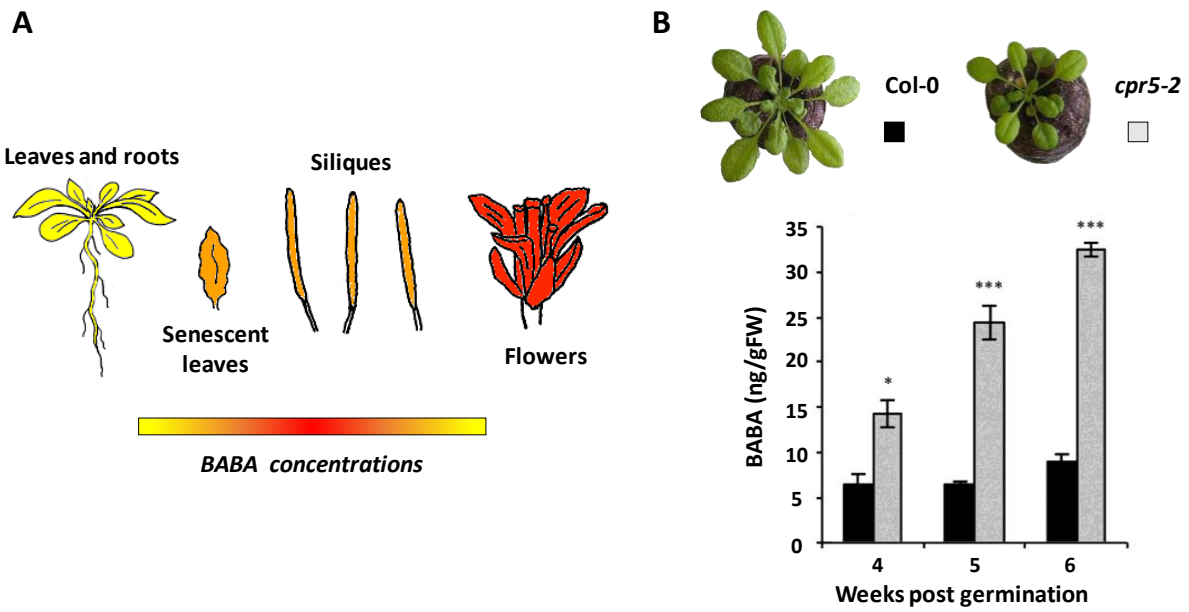

Fig. 1-11: Pattern of accumulation of BABA in Arabidopsis and in the BABA-accumulating mutant *cpr5-2*. A: accumulation of BABA in leaves and roots, senescent leaves, siliques, and flowers. Images of plant organs were obtained from the Klepikova Atlas of arabidopsis.org. B: BABA concentrations in Col-0 wild-type and *cpr5-2* mutant at 4-, 5- and 6-weeks post-germination.

1.4. References

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2. Aim of the project

Francesco Stefanelli, Ivan Baccelli and Brigitte Mauch-Mani

Although the knowledge of the physiology and biochemistry of BABA in plants has increased during the last years, different aspects keep unknown.

One point still unsolved is the BABA biosynthesis and the regulation of BABA accumulation during stresses. Only the patterns of accumulation of BABA are known up to now. Moreover, *cpr5-2* is the sole mutant characterized by altered BABA levels compared to the Col-0 wild type. Therefore, in this work will be used a transcriptomic approach to search genes involved in BABA metabolism in plants. For this purpose, there will be prepared RNA libraries from Arabidopsis Col-0 plants stressed with biotic and abiotic stresses and respective control plants. RNA libraries from untreated *cpr5-2* plants and Col-0 wild type control plants will be prepared as well. Subsequently, these libraries will be used for RNA-seq analysis, and there will be obtained genes differently expressed in common between the different treatments and *cpr5-2* mutant. We will then use T-DNA insertional lines of the genes upregulated in common for their capacity to accumulate BABA, to find mutants with altered BABA concentrations.

A second aspect of BABA physiology that this work will investigate is the relation existing between BABA and phytohormones. For this goal, Arabidopsis Col-0 plants will be treated with a series of plant hormone solutions sprayed or drenched into soil. Afterwards, the concentration of BABA would be analyzed searching for the hormone able to alter BABA concentrations. Finally, to confirm that the altered BABA concentration found could depend on the inducing-hormone signaling pathway, we will look at BABA levels in hormone-related mutants.

A third aspect that has not been explored yet is the subcellular localization of BABA. Experiment with sprayed and soil-drench ^{14}C-BABA on tomato, Arabidopsis and other plant species demonstrated that BABA moves both acropetally and basipetally. By contrast, subcellular localization is not well understood, as both cytoplasmic and parietal localizations have been observed. Therefore, we decided to clarify this topic using alkyne-tagged BABA molecules. We will incubate Arabidopsis seedlings in a solution of tagged-BABA for two days. Subsequently, tagged-BABA will be bind with the fluorophores Alexafluor 488 using copper-catalyzed cycloaddition. The molecule obtained can emit fluorescence when excited. Therefore, the localization of BABA may be visualized *in vivo* using confocal microscopy.

3. Searching genes involved in BABA metabolism in Arabidopsis

Francesco Stefanelli, Ivan Baccelli and Brigitte Mauch-Mani

Plants synthesize several non-protein amino acids and, among them, β-amino acids. Plants constitutively synthesize β-aminobutyric acid (BABA), and its concentrations increase after a stressful condition. BABA has also been employed as a priming agent and protects towards both biotic and abiotic stresses. Nevertheless, the metabolism of BABA in plants remains obscure. Therefore, we decided to perform an RNA-seq analysis to identify common genes expressed during various BABA-inducing stresses and in the mutant *cpr5-2* (a constitutive producer of high levels of BABA). We found ten genes upregulated in common and one downregulated between the different stresses and *cpr5-2*. Nevertheless, screening T-DNA mutants for the candidate genes, we have not found a mutant showing altered BABA concentrations compare to wild-type. New experiments have to be performed for obtaining new insights in BABA biosynthesis.

3.1. Introduction

Only twenty α-(L)-amino acids participate at the biosynthesis of proteins in plants. However, plants can synthesize a large number of amino acids not involved in proteinogenesis, the so-called non-protein amino acids (NPAAs) (Bell, 2003). NPAAs display several physiological roles, spanning from toxins to the storage of nitrogen (Bell, 2003; Vranova et al., 2011). Some NPAAs can also enter into the primary metabolism: for example, ornithine acts as a precursor of arginine and enters in the biosynthesis of polyamines as well (Alcázar et al., 2006; Kalamaki et al., 2009; Slocum, 2005). β-amino acids represent a class of NPAAs having the amino group in the β-carbon instead of the adjacent α-carbon, which is characteristic of the proteinogenic amino acids. Although rare in biological systems, β-amino acids enter in the composition of bacterial antibiotics, non-ribosomal peptides, and anticancer drugs (Kudo et al., 2014). Plants can also synthesize β-amino acids starting from α-amino acids, by reactions catalyzed by aminomutases (Feng et al., 2011; Yan et al., 2015). A recently discovered plant β-amino acid is BABA, one of the isomers of the aminobutyric acid along with GABA and AABA. BABA in plants is 60- and 1200-fold less concentrated than AABA and GABA, respectively (Thevenet et al., 2017). However, in contrast to GABA and AABA, exogenous BABA application induces priming and results effective against both biotic and abiotic stresses (Cohen and Gisi, 1994; Cohen et al., 1994; Cohen et al., 2016). Generally, plants accumulate BABA during salt stress and submergence. Furthermore, the accumulation of BABA occurs during biotic stress, such as infecting Arabidopsis with *Pst* DC3000 and with *P. cucumerina* (Thevenet et al., 2017). During both PTI, plants increase BABA concentrations. For example, BABA levels raised treating Arabidopsis Col-0 with flg22, while in the flg22-insensitive ecotype Ws-0, the flg22-dependent BABA induction was not observed (Baccelli et al., 2017). Avirulent *Pst* AvrRpt2 inoculation also increased BABA concentrations in Arabidopsis, earlier than infecting with the virulent strain *Pst* DC3000. These data suggested a BABA accumulation during ETI (Baccelli et al., 2017). Looking at the organ level, plants accumulate BABA mainly in flowers, developing seedlings, senescent leaves, and seeds; by contrast, leaves, seedlings, and roots are the sites of the lowest accumulation (Balmer et al., 2019).

Nevertheless, no biosynthetic pathways are known for BABA and neither BABA-signaling and BABA-interacting mutants have been discovered yet. Up to now, all the data concerning plant-produced BABA come from the quantification of BABA concentrations using a

chromatographic approach (Baccelli et al., 2017; Thevenet et al., 2017; Balmer et al., 2019). However, Balmer et al. (2019) discovered that the *cpr5-2* mutant synthesizes high levels of constitutive and induced BABA. *CPR5* encodes for a transmembrane nucleoporin physically associated with the nuclear pore complex (Gu et al., 2016). *CPR5* negatively regulates ETI and programmed cell death blocking the nucleocytoplasmic transport activity of nuclear signaling cargos (Gu et al., 2016). Furthermore, *CPR5* overexpressing mutant shows nuclear retention of the transcription factors ABI5, NPR1, and JAZ1 regulating different hormonal signaling (Gu et al., 2016). Consequently, the several mutant alleles associated with *CPR5* express pleiotropic phenotypes, such as accelerated leaf senescence, spontaneous cell death-treated lesions, altered trichome development, hypersensitivity to ABA and elevated levels of salicylic acid and jasmonic acid (Kirik et al., 2001; Jing et al., 2002; Yoshida et al., 2002; Heidel et al., 2004). Hence, it could be assumed that the higher concentrations of BABA found in *cpr5-2* mutant are related to the constitutive release of transcripts involved in BABA biosynthesis and regulation.

Therefore, according to this hypothesis, we have compared transcripts from *cpr5-2* with those derived from Arabidopsis plants exposed to different BABA-inducing-stresses, using an RNA-sequencing (RNA-seq) approach. We found ten genes expressed in common between the different treatments and *cpr5-2,* and one down-regulated, involved in various aspects of plant resistance to both biotic and abiotic stresses. Afterward, we investigated the BABA concentrations after salt stress in insertional T-DNA lines of the ten up-regulated genes. In this way, we wanted to test whether these mutants were impaired in BABA biosynthesis or accumulation. Unfortunately, we did not find mutants with altered BABA concentrations.

3.2. Materials and methods

3.2.1. Plant material and growth condition

For this work, we used 5-week-old *Arabidopsis thaliana* plants accession Columbia-0 (Col-0) multiplied from a stock purchased at Lehle Seeds, Round Rock, TX, USA. Constitutive-high-BABA levels mutant *cpr5-2* (Col-0 background) (Balmer et al., 2019) were multiplied from a stock purchased at NASC. Homozygous and segregating T-DNA insertional lines of the genes found with RNA-seq analysis (**Table S3-1**) were bought from NASC. Anton-Schnäffner from Helmotz Institute in Munich shared the T-DNA insertional line *utg76b1-1* (SAIL_1171A11) in Col-0 background. Homozygous T-DNA insertional line *SALK_110849*

in Col-0 background was genotyped using specific primers (forward primer CGAATGAGTTGGTAAGAAGGATG, reverse primer TCTAAGCACAACCAACCGATC).

Plants were sown on hydrated Jiffy-7 peat pellets and stratified at 4 °C in darkness for 2 days before being placed in controlled growth cabinets. Plants were cultivated under standard growth conditions: 21 °C day 18 °C night with 8h-day (light intensity of 100 μmol/m^2/s) and 16h-night (short-day-light conditions), at ~70 percent relative humidity until run-off. For seeds production, we grew plants at long-day-light conditions (16h-day and 8h-night, light intensity of 100 μmol/m^2/s) at the same temperatures and humidity of short-day-conditions.

3.2.2. Biotic and abiotic stress application

5-week-old Arabidopsis Col-0 plants were sprayed with a spore suspension (5×10^6 spores mL^{-1}) of *Plectosphaerella cucumerina* BMM in half-strength (12 $g.L^{-1}$) Potato Dextrose Broth (PDB/2, Sigma-Aldrich), and incubated in 100% relative humidity conditions until run-off. We used PDB/2 sprayed plants as control. Infection with *AvrRpt2* was performed infiltrating plants with a bacterial suspension of 10^7 cfu/mL in $MgCl_2$ 10 mM. As a control, we infiltrated plants with a solution of 10 mM $MgCl_2$. Plants used for submergence experiments were incubated in tap water until run-off (Thevenet et al., 2017). Salt stress was performed by drenching soil with a solution of 200 mM NaCl. For NaCl, submergence treatments and *cpr5-2* plants, we used Col-0 non-treated plants as control. For BABA and RNA extraction, the aerial part of the plants was harvested, frozen in liquid nitrogen and put at -80 °C.

3.2.3. Extraction and quantification of BABA

We extracted and quantified BABA using the protocol from Thevenet et al. (2017). The analyses were performed by the Neuchâtel Platform of Analytical Chemistry of the University of Neuchâtel. Differences in BABA concentrations between control and treated plants or *cpr5-2* were analyzed using Student's t-test. Statistical analysis was performed using Microsoft Excel.

3.2.4. Nucleic acid extraction

Plant RNA extraction was performed using the NucleoSpin® RNA Plant kit (Macherey Nagel, Düren, Germany). RNA was then treated with DNase (Macherey Nagel, Düren, Germany) and retro-transcribed into cDNA using SuperScript III RT (Invitrogen, Waltham,

MA, USA). RNA integrity was measured with Agilent Fragment Analyzer (Agilent Technologies, Santa Clara, CA, USA). Genomic DNA was extracted using Quick DNA Prep for PCR procedure used according to Glazebrook and Weigel (2002).

3.2.5. RNA-seq analysis

Purity-filtered reads were adapters and quality trimmed with Cutadapt (V1.8, Martin, 2011). Reads matching to ribosomal RNA sequences were removed with fastq_screen (v. 0.11.1). Remaining reads were further filtered for low complexity with reaper (v. 15-065, Davis et al., 2013). Reads were aligned against *Arabidopsis thaliana* (version TAIR10) genome using STAR (v. 2.5.3a, Dobin et al., 2013). The number of read counts per gene locus was summarized with HTSEQ-count (v. 0.9.1, Anders et al., 2015) using *Arabidopsis thaliana* gene annotation (Ensembl Plants version 39). Quality of the RNA-seq data alignment was assessed using RSeQC (v. 2.3.7, Wang et al., 2012). Statistical analysis for RNA-seq for genes was performed in R (R version 3.4.3). Genes with low counts were filtered out according to the rule of 1 count per million (CPM) in at least 1 sample. Library sizes were scaled using Trimmed Mean of M-values (TMM) normalization and log-transformed CPM (EdgeR package version 3.20.8; Robinson and Oshlack, 2010). Next, quantile normalization was applied using normalize BetweenArrays (Limma package) for correcting the bias of the *AvrRpt2* group. Differential expression was computed with limma-trend approach (Ritchie et al., 2015) by fitting the selected samples into one linear model. According to the model, contrasts were tested using a moderated F-test. The adjusted p-values were computed by the Benjamini-Hochberg method, controlling for false discovery rate (FDR or adj.P.Val). Successively, three independent linear models were performed. In the first linear model, we did the following contrasts: NaCl versus Col-0, Submergence versus Col-0 and *cpr5-2* vs Col-0. In parallel, two other linear models were made, performing the following contrasts: *P.cucumerina* vs PDB/2 and *AvrRpt2* vs $MgCl_2$. For all the linear models, a moderated F-test was used. The adjusted p-value is computed by the Benjamini-Hochberg method, controlling for false discovery rate (FDR or adj.P.Val) cut-off 0.05. Gene lists using significant genes (FDR 5% threshold) were crossed to detect the gene set of intersection between the 3 models.

RNA-seq analysis were performed at the Lausanne Genomic Technologies Facility of the University of Lausanne.

3.2.6. Expression pattern analysis

We employed *Arabidopsis* AtGenExpress eFP from ePlant (http://bar.utoronto.ca/eplant) (Waese et al., 2017) to obtain preliminary data on the expression pattern of AT1G13830, AT1G30700, *AtMYB45*/AT3G48920 and AT4G11170 for tissues development and for anoxia and salt stress, and for *avrRpm1* and *B. cinerea* infection. Details of stress application, growth condition, harvesting time and statistical analysis we remind to Waese et al. (2017). Concerning the expression pattern of AT5G38865 we employed the Klepikova atlas from ePlant (http://bar.utoronto.ca/eplant) (Klepikova et al., 2016).

3.3. Results

3.3.1. RNA sequencing on plants challenged with BABA-inducing stresses and in the constitutive-high-BABA-levels mutant *cpr5-2*

To get further information on the genes playing a role in BABA biosynthesis, signaling, and regulation in plants, we decided to perform a RNA-seq analysis. RNA libraries were prepared starting from 5-week-old *Arabidopsis thaliana* Col-0 plants exposed to different stresses that are known to induce high levels of BABA. We chose a 200 mM NaCl solution drenched in soil and submergence as abiotic stresses, with mock-treated Col-0 plants as control. Infections with the *Pst* AvrRpt2 and with *P. cucumerina* BMM were selected as biotic stresses, with respectively 10 mM $MgCl_2$-treated and PDB/2-sprayed plants as controls. We also prepared an RNA-seq library for the constitutive high BABA-levels mutant *cpr5-2*, using mock wild-type Col-0 plants as control. We used three biological replicates for each treatment, each one composed of a pull of ten different plants, for a total of 24 samples. Samples ID and names are summarized in **Table S3-2**. Total RNA was extracted before BABA levels reach the peak of induction reported in the literature, considering that generally transcript concentrations increase before the protein and metabolite accumulation(Baccelli et al., 2017; Thevenet et al., 2017; Balmer et al., 2019). Therefore, we harvested plant material 18 hours post-treatment (hpt) for NaCl-treated and for *AvrRpt2*-infected plants, 24 hpt for submergence-treated and 36 hpt for *P. cucumerina*-infected plants. Total RNA from *cpr5-2* was extracted contemporary with mock-treated Col-0 control from abiotic stresses.

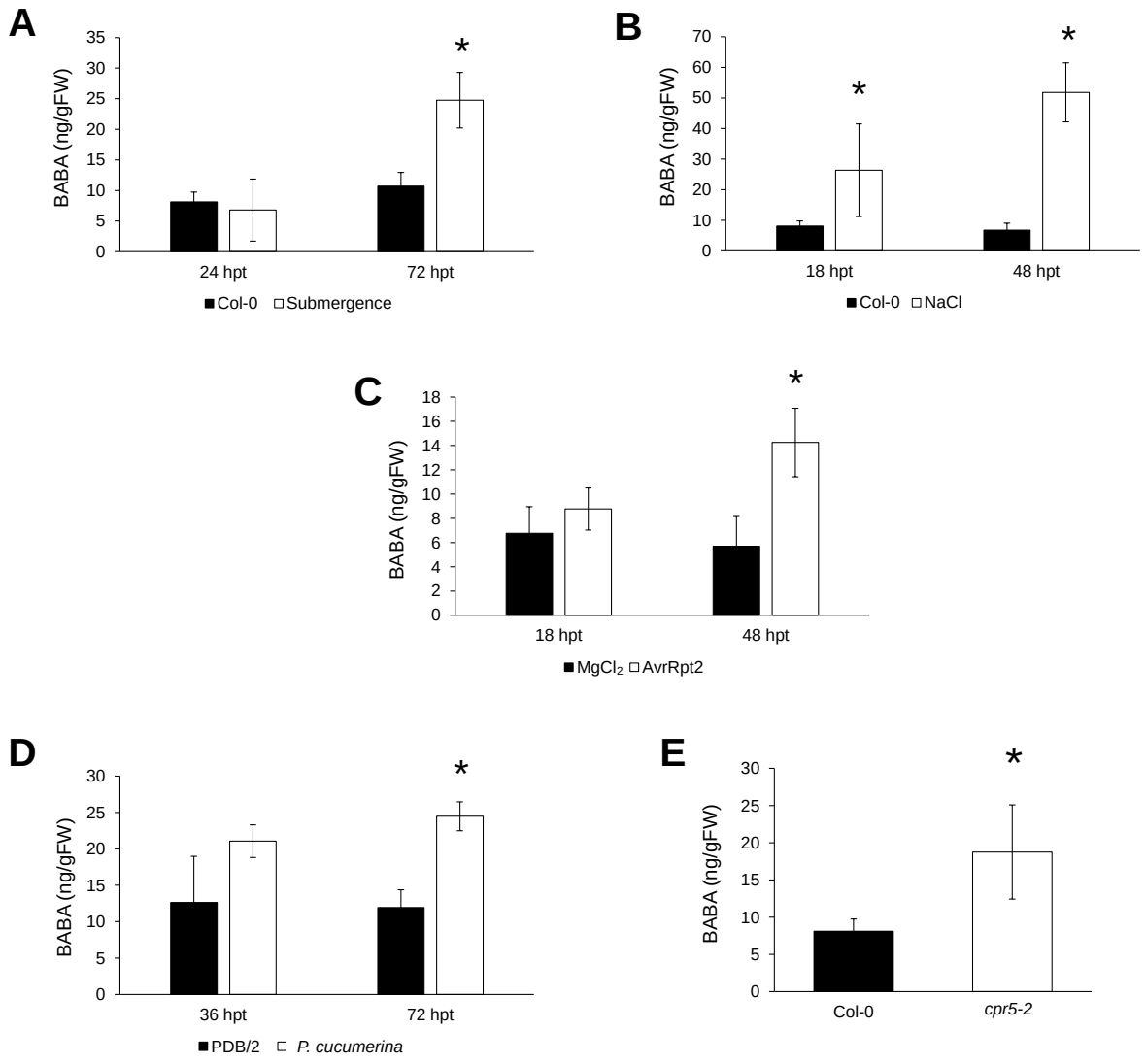

Fig. 3-1: BABA concentrations at the time of RNA library preparation in plants treated with different BABA-inducing stresses and in *cpr5-2* mutants. A: Mock-treated and submerged 5-week-old Arabidopsis Col-0 plants were harvested and processed for extracting BABA at 24 hpt and at 72 hpt as reported in Thevenet et al. (2017). **B**: 200 mM NaCl-treated and mock-treated 5-week-old Arabidopsis Col-0 plants were harvested and processed for extracting BABA at 18 hpt and at 48 hpt. **C**: leaves of 5-week-old Col-0 plants infiltrated with 10 mM $MgCl_2$ as control and with a 10^7 cfu/mL suspension of AvrRpt2 in 10 mM $MgCl_2$ harvested and processed for extracting BABA at 18 hpt and 48 hpt. **D**: 5-week-old Col-0 plants sprayed with PDB/2 as control and with a suspension of 5 x 10^5 spores/mL suspension of *P. cucumerina* in PDB/2 harvested and processed for extracting BABA at 36 hpt and 72 hpt. **E**: BABA concentration in 5-week-old *cpr5-2* mutants compared to 5-week-old Col-0 control plants. Data represent the mean and standard deviation (n = 3 biological replicates). All experiments were repeated in triplicate. Asterisks indicate significant differences as determined by a Student t -test: *P < 0.05.

Prior to further analysis, we investigated the levels of BABA in each sample used for preparing the RNA libraries. Moreover, we analysed the BABA levels at the time of the peak in concentrations reported in the literature in both treated and control plants. In submergence-treated plants, BABA concentrations at 24 hpt were similar to control, while at 72 hpt BABA significantly increased (**Fig. 3-1A**). In salt stress-treated plants, we found a slight induction

compared to control Col-0 plants at 18 hpt, increasing then at 48 hpt (**Fig. 3-1B**). In both *Pst* AvrRpt2- and *P. cucumerina*-infected plants, BABA concentrations did not increase after 18 hpt and 36 hpt, but at 48 and 72 hpt were significantly induced compared to control in both treatments (**Fig. 3-1C** and **3-1D**). Finally, c*pr5-2* mutant showed a significant induction of BABA compared to control plants as well (**Fig. 3-1E**).

Afterward, we performed the RNA-seq analysis with the libraries previously prepared. More than 20 million raw RNA-seq reads were generated in each sample, excluded for sample number 05 from NaCl-treated plants with 15 million (**Fig. S3-1A**). Gene coverage profiles were not similar for all replicates. Whereas most of the samples looked uniform, sample 20 from PDB/2-treated plants and sample 23 from *P. cucumerina*-infected plants showed a marked 3' end bias that could be related to the lower RNA integrity (**Fig. S3-1B**). Likewise, other samples displayed 3' end bias to some extent. However, the samples belonging to plants infected with *AvrRpt2* showed fewer transcripts compared to the other samples (**Fig. S3-1C**). Therefore, samples were normalized using TMM followed by quantile normalization.

Differential expression was computed with limma trend approach (Ritchie et al., 2015), by fitting samples in three different linear models. The first linear model included total RNA from plants treated with NaCl and submergence, more the mutant *cpr5-2* each one confronted with the Col-0 control samples. Therefore, plants treated with NaCl showed 1069 genes up-regulated and 1107 down-regulated, while in submergence-stressed plants showed 3398 down-regulated and 2662 up-regulated (**Table 3-1**) (**Supplementary file**). *Cpr5-2* showed 730 genes down- and 840 upregulated compared to Col-0 (**Table 3-1**). Crossing the up-regulated genes found in NaCl, submergence and *cpr5-2* we found 15 genes in common (**Fig. 3-2A**), while crossing down-regulated genes we discovered 3 genes (Fig. **3-2C**). The second linear model, including material from the AvrRpt2 infected plants confronted with $MgCl_2$ control group, found 10841 genes differentially expressed, with 5476 (**Table 3-1**). Finally, the third group composed of samples of the library of *P. cucumerina* infected plants versus the PDB/2 control group discovered 8873 genes differently expressed, with 4599 down-regulated and 4274 up-regulated (**Table 3-1**) (**Supplementary file**). Genes with an FDR threshold of 5% were crossed to detect the gene set of intersection between the 3 models, resulting in 10 genes up-regulated in common (**Fig. 3-2B**) and one down-regulated (Fig. **3-2D**) (**Supplementary file**). The expression for the up-regulated genes in both $MgCl_2$ and AvrRpt2 groups was higher compared to that of the other groups (**Fig. 3-3**).

	NaCl vs Col-0	Submergence vs Col-0	*cpr5-2* vs Col-0	AvrRpt2 vs $MgCl_2$	*P. cucumerina* vs PDB/2
Down-regulated	1107	3398	730	5476	4599
Up-regulated	1069	2662	840	5365	4274
Not significant	18080	14196	18686	9415	11383

Table 3-1: Post-hoc classification of the genes expressed in the different contrasts in down-regulated, up-regulated and not significantly expressed genes.

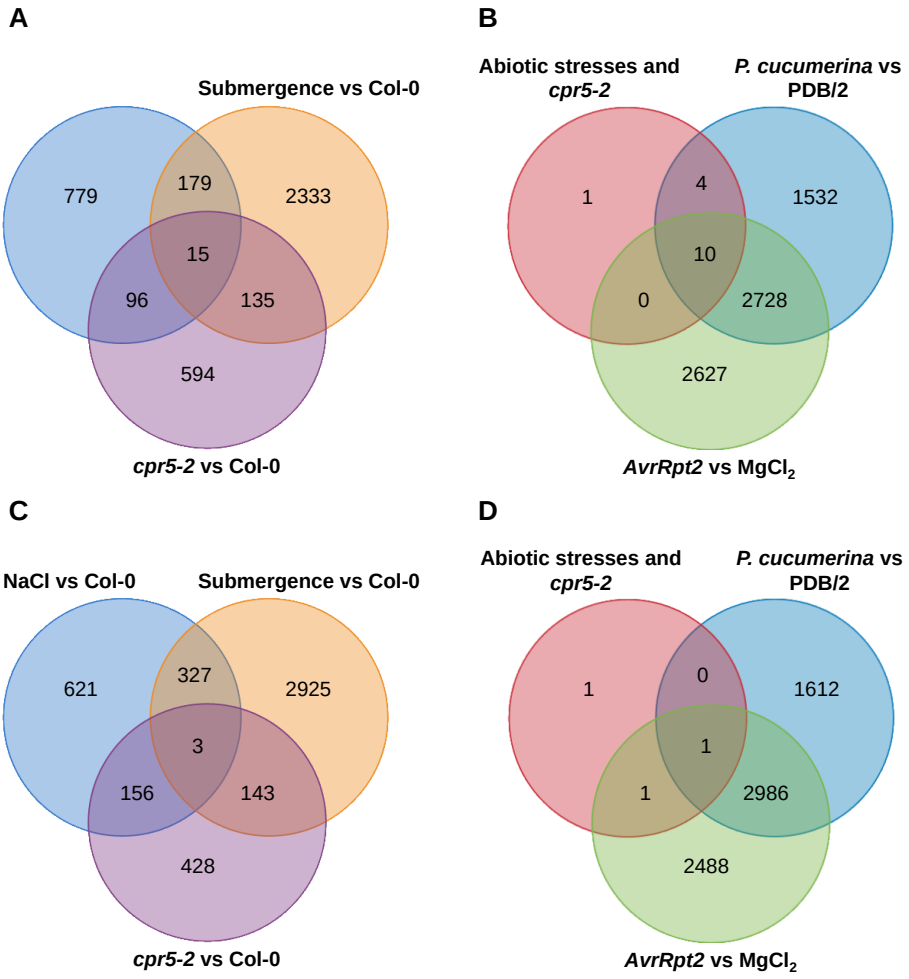

Fig. 3-2: Venn diagram of the genes found with RNA-seq up-regulated and down-regulated in common among BABA-inducing stresses-treated Arabidopsis Col-0 and *cpr5-2*. A: Venn diagram showing the number of up-regulated genes found with RNA-seq (FDR 5% threshold) of the first linear model, built intersecting the NaCl vs Col-0, submergence vs Col-0 and *cpr5-2* vs Col-0 constrasts. **B:** Venn diagram showing the number of up-regulated genes found with RNA-seq (FDR 5% threshold) intersecting the up-regulated genes found in common in the first linear model between abiotic stresses and *cpr5-2*, and the second and third linear models composed of *P. cucumerina* vs PDB/2 contrast and AvrRpt2 vs $MgCl_2$ contrast. **C:** Venn diagram showing the number of down-regulated genes found with RNA-seq (FDR 5% threshold) of the first linear model, built intersecting the NaCl vs Col-0, submergence vs Col-0 and *cpr5-2* vs Col-0 contrasts. **D:** Venn diagram showing the number of down-regulated genes found with RNA-seq (FDR 5% threshold) intersecting the up-regulated genes found in common in the first linear model between abiotic stresses and *cpr5-2*, and the second and third linear models composed of *P. cucumerina* vs PDB/2 contrast and *AvrRpt2* vs $MgCl_2$ contrast.

Of the 10 genes up-regulated in common between the different stresses and the cpr5-2 mutant found, only six have been already characterized. Among the characterized genes, we found *plasmodesmata germin-like protein 5* (*GLP5 – AT1G09560*), *korrigan 2* (*KOR2 – AT1G65610*), *kunitz trypsin inhibitor 1* (*KTI1 – AT1G73260*), *pectin methylesterases 17* (*PME17 – AT2G45220*), *UDP-dependent glycosyltransferase 76B1* (*UGT76B1 – AT3G11340*) and the *A. thaliana class B heat shock factor B1* (*HSFB1 – AT4G36990*) (**Fig.3-3**). Four genes up-regulated and one down-regulated gene has an unknown function in Arabidopsis. These are *AT1G13830, AT1G30700, AtMYB45/AT3G48920,* and *AT4G11170* for the up-regulated and *AT5G38865* for the down-regulated genes (**Fig. 3-3**).

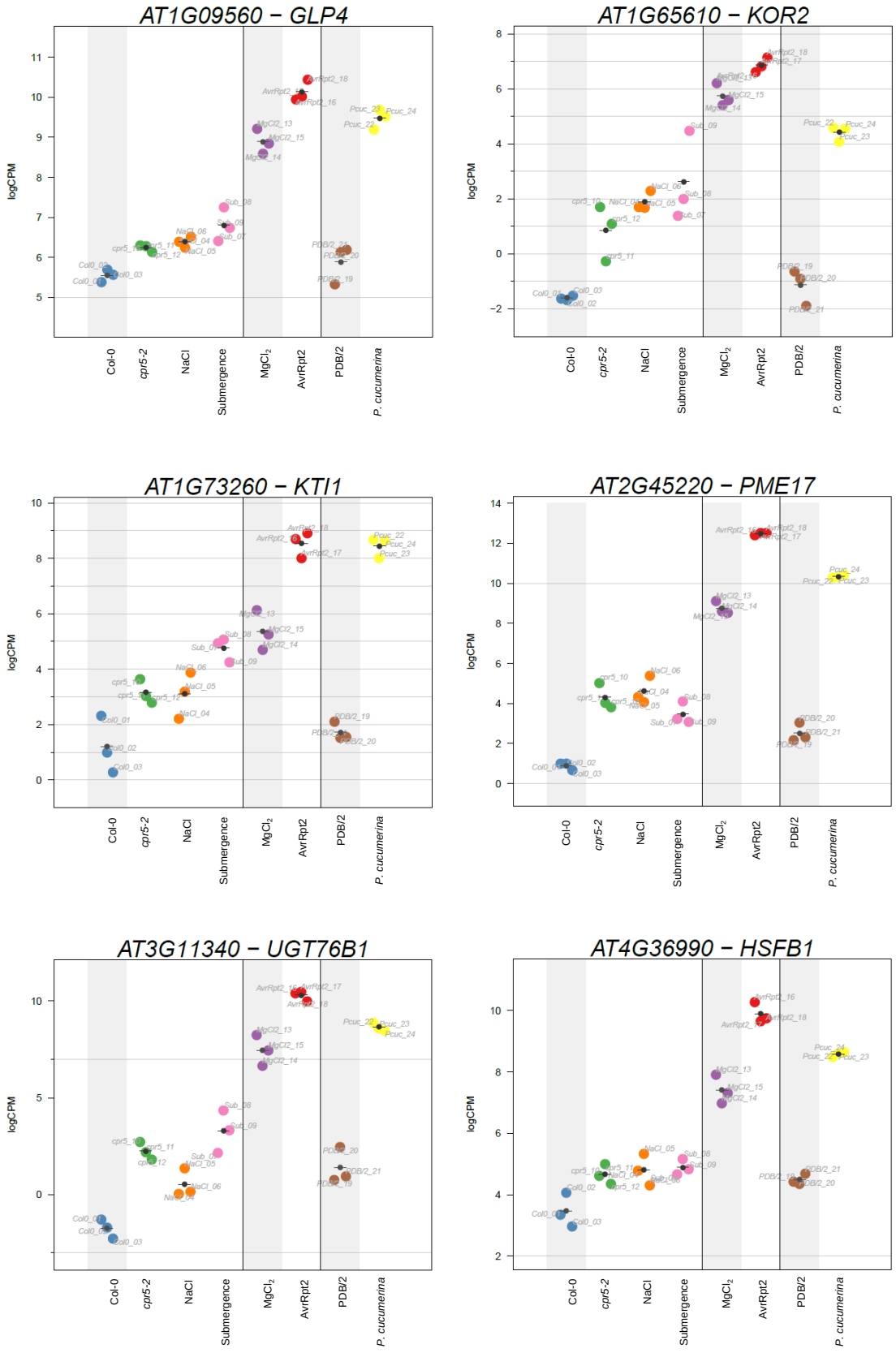

Fig. 3-3 (Part I): Differential expression of the genes found with RNA-seq expressed in common between the different stresses applied and in *cpr5-2*. Each sample is marked with its Sample ID and Sample name and with different colors depending on the group they belong to. Groups have been placed on the horizontal axis. A sharper vertical line separates the three linear models. Gene expression is defined in log counts per million (logCPM).

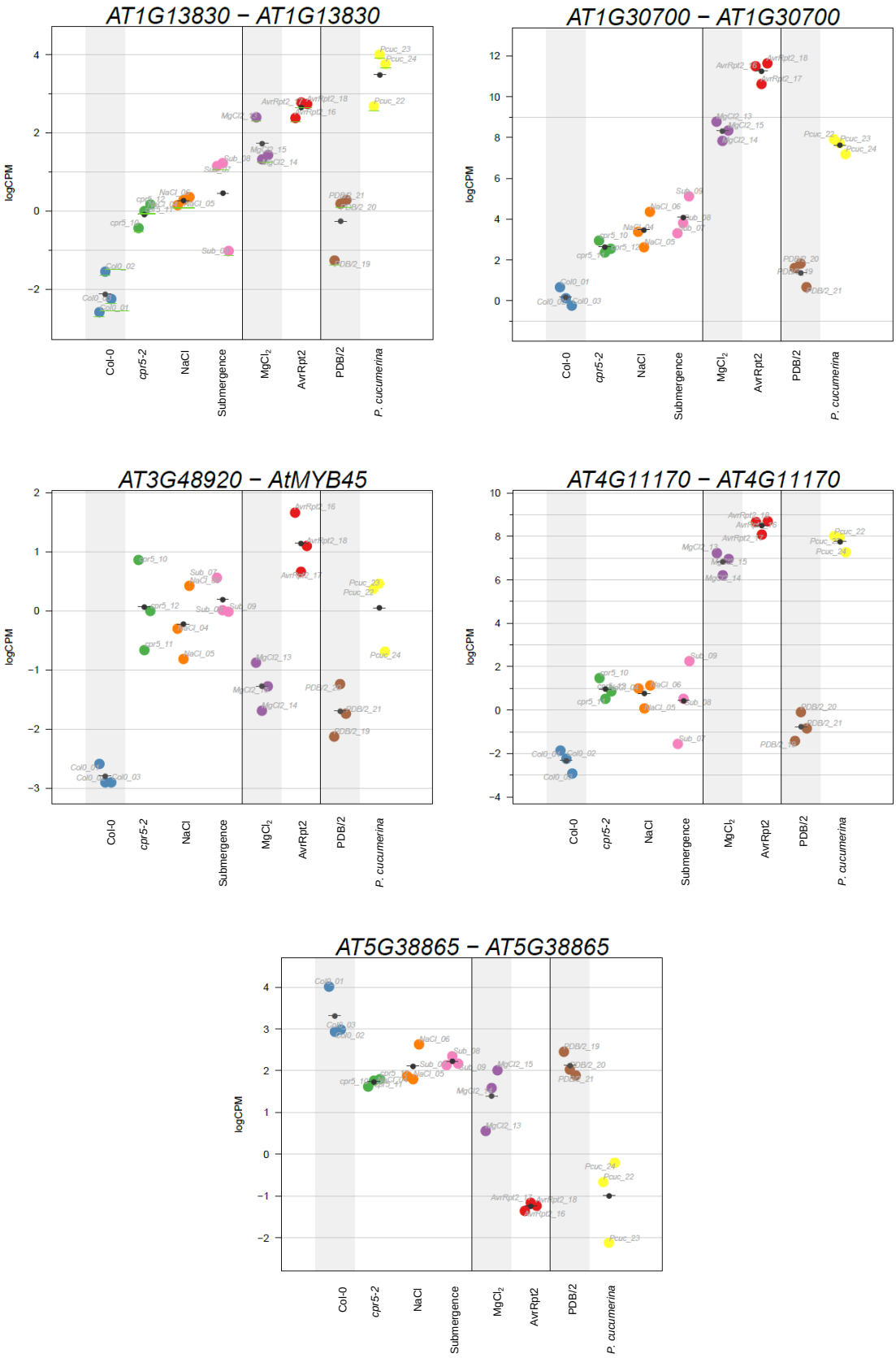

Fig. 3-3 (Part II): Differential expression of the genes found with RNA-seq expressed in common between the different stresses applied and in *cpr5-2*. Each sample is marked with its Sample ID and Sample name and with different colors depending on the group they belong to. Groups have been placed on the horizontal axis. A sharper vertical line separates the three linear models. Gene expression is defined in log counts per million (logCPM).

3.3.2. Comparison of the expression patterns of uncharacterized genes with public available resources

eFP Browser data (eFP at bar.utoronto.ca/eplant) is a publicly open platform for obtaining preliminary information about gene expression. We decided to compare our data for the uncharacterized genes with those available in eFP browser concerning NaCl treatment and anoxia, a submergence-induced stress (Lee et al., 2011), and with *Pst* avrRpm1 and *Botritis cinerea*. By this way we wanted to obtain preliminary data about the pattern of expression of these genes

eFP browser data showed that AT1G13830 is highly concentrated in seeds, developing siliques, second internode, and senescent leaves (**Fig. 3-4A**). During salt stress, AT1G13830 was down-regulated in roots, and no induction occurred in leaves, in contrast with our results (**Fig. 3-4B**). Similar contrasting data are present in anoxia-treated plants where there was no induction (**Fig. 3-4C**). *Pst* avrRpm1 infiltrated leaves data showed a slight induction compared to control (**Fig. 3-4D**). Finally, *B. cinerea*-infected leaves expressed a high induction after 48 hpt, similar as we obtained with *P. cucumerina* (**Fig. 3-4E**).

AtMYB45/AT3G48920 is a member of the *MYB transcription factors* gene family (Yanhui et al., 2006). Public data showed a high induction of the gene in the hypocotyl and slightly in flowers (**Fig. S3-2A**). *AtMYB45* expression did not change during salt stress but decreases during anoxia (**Fig. S3-2B** and **S3-2C**). During avirulent infection eFP browser data showed a quite strong induction at 24 hours, similar as we found with AvrRpt2 (**Fig. S3-2D**). Finally, leaves of *B. cinerea*-infected leaves seemed to accumulate high levels of *AtMYB45* transcripts after 48 hpt compared to control (**Fig. S3-2E**). At 18 hpt the gene was highly expressed in PDB/2-treated plants. However, transcripts increased in concentration in *B. cinerea* infected leaves in the time frame showed (**Fig. S3-2E**).

AT4G11170 seemed to be mainly accumulated in mature seeds and flowers and slightly in leaves (**Fig. S3-3A**). AT4G11170 was accumulated after salt stress in roots but not in leaves, while in anoxia, there was no induction (**Fig. S3-3B** and **S3-3C**). Infection with avirulent *Pst* avrRpm1 did not increase transcript levels (**Fig. S3-3D**). *B. cinerea* also induced AT4G11170 at 48 hpt (**Fig. S3-3E**).

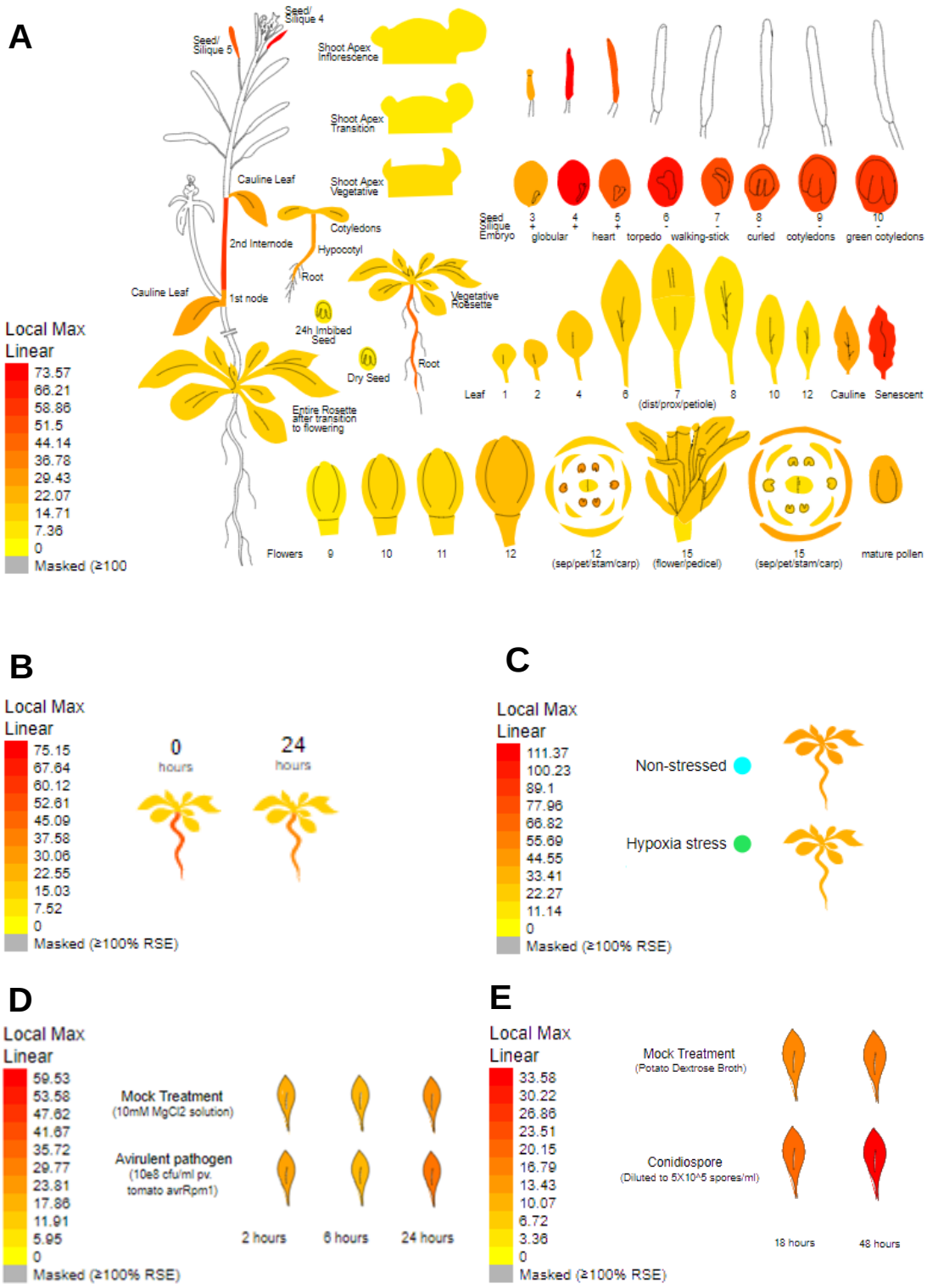

Fig. 3-4: Pattern of expression of AT1G13830, according to efp Browser. A: Anatomical map of Arabidopsis generated with the AtGenExpress eFP (bar.utoronto.ca/eplant) and modified for this work showing the pattern of expression of AT1G13830. **B:** Pattern of expression of AT1G13830 in 18-day-old wild-type Col-0 plants treated with 150 mM NaCl and harvested at time 0 and 24 hpt. **C:** Pattern of expression of AT1G13830 in 7-day-old Arabidopsis Ler plants cultivated on MS agar plates and grown for 12 hours with argon gas until run-off. **D:** Pattern of expression of AT1G13830 in leaves of 5-week-old Arabidopsis Col-0 plants infiltrated with 10 mM $MgCl_2$ and with a suspension of 10^8 cfu/mL of *Pst* AvrRpm1. **E:** Pattern of expression of AT1G13830 in 4-week-old Arabidopsis Col-0 drop-inoculated with a 5 x 10^5 spores/mL *B. cinerea* suspension and with PDB/2 broth. Lower expression strength is expressed by the color yellow, while higher is expressed in red. Images were generated with the AtGenExpress eFP (bar.utoronto.ca/eplant).

eFP browser data showed strong induction of AT1G30700 in mature seeds and a slight induction in senescent leaves (**Fig. S3-4A**). Roots but not leaves of salt-stressed plants induced the gene, while hypoxia seemed to up-regulate the expression of the gene (**Fig. S3-4B** and **S3-4C**). Avirulent infiltration seemed to highly induce AT1G30700 compared to $MgCl_2$-infiltrated leaves, similar to our results (**Fig. S3-4D**). The necrotrophic pathogen also induced AT1G30700 transcripts 48 hpt, as we found with *P. cucumerina* (**Fig. S3-4E**).

Klepikova eFP browser (eFP at bar.utoronto.ca/eplant) (Klepikova et al., 2016) (**Fig. S3-5**) showed that a high expression had been detected only in stamen for the down-regulated gene AT5g38865. No data are available for the stresses similar to those applied in this work (**Fig. S3-5**).

Finally, the preliminary data from eFP Browser found that the up-regulated genes were highly expressed in BABA accumulating organs, such as flowers, seeds and senescent leaves. Furthermore, all the genes were highly expressed during *B. cinerea* infection, while only some were expressed after *Pst avrRpm1* infection. Finally, the genes upregulated during salt-stress showed accumulation mainly in roots. During hypoxia two genes were upregulated while only *MYB45* showed a downregulation in contrast to the data in our work from submergence.

3.3.3. Analysis of BABA concentrations in T-DNA-insertional lines of the commonly up-regulated genes found with RNA-seq between the different stresses and *cpr5-2*

To confirm that the up-regulated genes found with RNA-seq played a role in BABA metabolism in Arabidopsis, we drenched the soil with NaCl solution, and we analysed at BABA concentrations in T-DNA-insertional lines of the previously mentioned genes (**Table S3-1**). For the segregating T-DNA lines of *AT1G13830, AT1G30700,* and *AT4G11170* (**Table S3-1**), we looked at BABA concentrations in each individual of the population, and we cut only some leaves without killing the plant for further genotyping. As negative and positive controls, we used mock and NaCl-treated Col-0 wild-type plants. All homozygous mutants tested showed wild-type induced concentrations of BABA after salt stress (**Fig. 3-5A** and **3-5B**). In the SALK_110849 line of the AT1G13830 gene, six individuals (number 1, 13, 33, 34, 40 and 42) did not differ from mock-treated plants (**Fig. S3-6**). The treated population did not survive to salt stress to reach reproductive stages, and plant-frozen material was not enough to perform a DNA extraction for genotyping. Therefore, we re-sowed the population, and we selected

homozygous mutants of the plants to confirm if the low-induced BABA concentrations phenotype was an effect of the insertion in the gene. Nevertheless, the homozygous line of SALK_110849 showed wild-type levels of BABA after NaCl treatment, as observed in the Col-0 wild-type (**Fig. 3-4C**). Finally, in NaCl-treated SALK_117810 and SAL_112198 T-DNA insertional lines, each individual did not differ from NaCl-treated wild-type in BABA concentrations (**7** and **Fig. S3-8**).

The lines used in this work for the genes upregulated in common between the BABA-inducing stresses and in the *cpr5-2* mutant showed wild-type levels of BABA accumulation after salt stress, impeding thus up to now to correlate these genes with BABA metabolism.

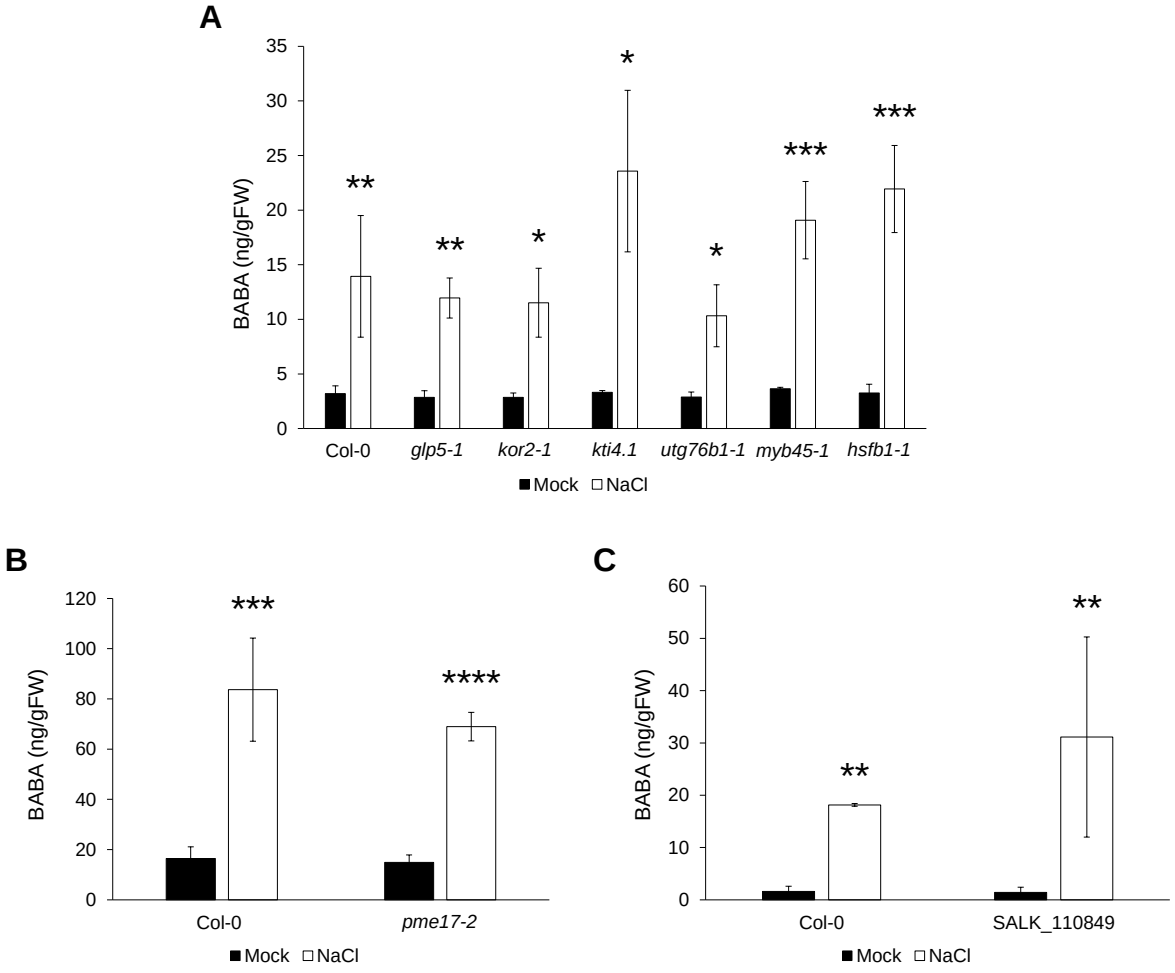

Fig. 3-5: BABA concentration in T-DNA insertional lines of the genes found with RNA-seq up-regulated in common between the different stresses applied and *cpr5-2*. A and B: BABA concentrations in T-DNA homozygous lines for some of the genes expressed in common among the different stresses and *cpr5-2* 48 hpt with a soil-drench 200 mM NaCl solution. All experiments were repeated twice. **C**: BABA concentrations in the T-DNA homozygous line SALK_110649 48 hpt with a soil-drench 200 mM NaCl solution. All experiments were repeated three times. Asterisks indicate significant differences as determined by a Student t -test: *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

3.4. Discussion

BABA is also a plant metabolite, mainly accumulated in senescent tissues, seeds, and flowers. Furthermore, plants increase the concentrations of BABA after both biotic and abiotic stress (Thevenet et al., 2017; Balmer et al., 2019). Nevertheless, neither the biosynthetic pathway nor the regulation of BABA accumulation is known yet. This work tried to elucidate these aspects of BABA physiology using a transcriptomic approach.

For this purpose, we decided to look at the common transcriptomic profile of Arabidopsis Col-0 plants treated with two abiotic stresses (soil-drenched NaCl solution and submergence) and with two biotic stresses (*P. cucumerina* and AvrRpt2 infection), known to increase BABA concentrations. Also, we analysed the transcriptome of the constitutive high-BABA levels mutant *cpr5-2*. For stressed plants, we extracted total RNA before the peak in induced-BABA concentrations. The rationale behind this is that BABA-related genes may follow the "signal delay" model for the relationship between mRNA and proteins (Liu et al., 2016). The "signal delay" model affirms that after a stimulus, mRNA concentrations increase and reach a peak. Afterward, the translated protein levels increase concomitantly with a decrease in transcripts concentrations (Liu et al., 2016). To corroborate our hypothesis, we firstly checked the concentrations of BABA at the time of RNA extraction and the peak of BABA concentrations reported in the literature. All stressed plants reported a wild-type concentration of BABA at the time of RNA extraction (**Fig. 3-1**), except for the NaCl-treated Arabidopsis that showed a slight induction at 18 hpt (**Fig. 3-1**), although less intense compared to that observed at 48 hpt (**Fig. 3-1**). A triggering induction during salt stress is typical of other amino acids involved in the response to abiotic stress. A classic example is proline, which works as an osmolyte to prevent water loss (Verslues and Sharma, 2010). Therefore, plants may accumulate BABA during salt stress as an osmolyte as well. Another possibility may be that BABA acts as a fast triggering signal during salt stress to start a quick response to this stress.

Successively, the different RNA libraries were employed for RNA-seq analysis. The analysis was conducted clustering the groups in three linear models. Each linear model was characterized by a specific control group. Indeed, in the first linear model, we grouped all the groups having the Col-0 group as control: NaCl, submergence and *cpr5*-2. The intersection of the different contrasts found 15 genes up-regulated in common and 3 down-regulated (**Fig.3-2A** and **Fig. S3-2A**). Comparing these 15 genes with those expressed in common between the second linear model (AvrRpt2-$MgCl_2$ contrast), and the third linear model (*P. cucumerina*-

PDB/2 contrast), we found 10 genes up-regulated in common between all stresses and *cpr5-2* (**Fig. 3-2B**). By the side, we found only one gene down-regulated in common (**Fig. 3-2D**). Interestingly, all the up-regulated genes in $MgCl_2$-treated plants looked highly expressed compared to other control groups, although to a lesser extent compared to *Pst* AvrRpt2 treated leaves (**Fig. 3-3**). This phenomenon may be caused by infiltrating leaves with a syringe.

Looking at their role in plant physiology, we found three genes playing a role in senescence and in plant-pathogen interaction as well. *KTI1/KTI4* has been identified as a leaf senescence-associated gene in a large-scale identification analysis using suppression subtractive hybridization assay (Gepstein et al., 2003). *KTI1/KTI4* codes for a proteinase inhibitor belonging to the *Kunytz Trypsin Inhibitors* (*KTIs*) gene family. Indeed, KTI1 can inhibit different proteinases, such as trypsin, chimotripsin, papain and chetepsin (Arnaiz et al., 2018). Using the same technique as Gepstein et al. (2003), Li et al. (2008) identified *KTI1* among the genes up-regulated by the necrotrophic pathogen *Erwinia carotovora* sub. *carotovora* elicitors in Arabidopsis. In our work, the induction during *P. cucumerina* infection of *KT1* suggests that KTI may play a role in plant-necrotrophic pathogen interaction. Furthermore, when infected with the avirulent strain of *Pst* carrying *avrB1*, the *KTI1* overexpressor mutant showed less bacterial-derived damages compared to wild-type and RNAi mutant (Li et al., 2008). By the side, public microarray data from Genevestigator showed that *KTI1* transcript concentrations increased in Arabidopsis after *Pst* DC3000 infection. Moreover, a strong induction was found in plants incubated with an avirulent strain carrying *avrRpm1*, similarly as we found in *Pst* AvrRpt2-infected plants (Zimmermann et al., 2004; Li et al., 2008). By the side, we found that *KTI1* is induced during abiotic stress as well. Other works found similar results in other plant species, suggesting a role of the *KTI* gene family during osmotic stress. Indeed, using comparative 2D-PAGE analysis, Lopez et al. (1994) found a salt inducible KTI protein family member in radish, named P22. Furthermore, Annamalai and Yanagihara (1999) discovered that the cabbage *KTI* gene member *BocHS* was induced by drought and heat stress, but not by salt nor ABA.

Another of the genes found in this work involved in senescence and plant-pathogen interaction as well is *HSFB1*. *HSFB1* is the most expressed member of the *class B- Heat Shock Factors* (*HSFBs*) gene family in *Arabidopsis thaliana* and is highly induced during senescence (Busch et al., 2005; Breeze et al., 2008). HSFB1 is a negative regulator of heat shock proteins and *HSFs* genes during non-heat stress conditions. At the same time, it works as a positive regulator of the same genes during acquired thermotolerance (Ikeda et al., 2011). Moreover,

HSFB1 acts as a negative regulator of JA- and ethylene-responsive genes *PDF1.2a* and *PDF1.2b* (Kumar et al., 2009). Interestingly, *Pst* AvrRpt2 infection induces *HSFB1* in both local-infected and distal leaves (Pick et al., 2012), confirming thus the results reported in this work and the importance of this gene in the onset of ETI. Moreover, HSFB1 negatively regulates SA-mediated gene transcription. Furthermore, SA-dependent growth repression did not occur in the mutant allele of *HSFB1 tbf1*, suggesting a role of HSFB1 in growth to defense transition. *Tbf1* mutant also fails to express elf18-triggered immunity and to secrete antimicrobial proteins, such as PR-1 (Pajerowska-Mukhtar et al., 2012). Finally, the GCN2-eIF2α regulating pathway regulates the transcription of *HSFB1*, which in turn regulates the ABA signaling during the pre-invasive stage of bacterial infection (Liu et al., 2019).

In contrast to *KTI1/KTI4* and *HSFB1*, *UGT76B1* is a negative regulator of senescence. Indeed, the senescence marker *SAG13* is highly up-regulated in the *ugt76b1-1* knockout mutant, which shows also an early senescence phenotype (von Saint Paul et al., 2011). *UGT76B1* is one of the members of the *UDP-dependenat glycosyl transferases* (*UGTs*) gene family that catalyzes the transfer of carbohydrates from a sugar donor to a small molecular acceptor. These enzymes are required for the biosynthesis of signaling molecules or secondary metabolites (Bowles et al., 2006). In particular, UGT76B1 catalyzes the glycosylation of isoleucic acid (ILA) and SA (von Saint Paul et al., 2011; Noutoshi et al., 2012; Maksym et al., 2018). ILA application induces resistance against *P. syringae* and inhibits root growth in a concentration-dependent manner (von Saint Paul et al., 2011). GUS-staining experiments and fluorescence images of GFP-tagged transgenic lines showed that *UGT76B1* is strongly expressed in young and lateral roots, in sepals, style, and young leaves (von Saint Paul et al., 2011). Public expression databases showed that *UGT76B1* the highest induced *UGT* member in both biotic and abiotic stresses. Other data showed that wounding and infection with *Pst* AvrRpt2 are both able to induce *UGT76B1*, confirming thus our results (Toufighi et al., 2005; von Saint Paul et al., 2011). However, infected with both *Pst* DC3000 and with *Pst* AvrRpt2, *ugt76b1-1* showed enhanced resistance compare to wild-type and overexpressor mutant (von Saint Paul et al., 2011). Furthermore, the *ugt76b1-1* knockout mutant showed reduced susceptibility to necrotrophic fungus *Alternaria brassicicola*, whereas overexpressor mutants showed an opposite phenotype. Therefore, knock out mutant expressed elevated levels of free glycosylated SA, and up-regulation of SA-marker genes (e.g., *PR1*, *PAD4*, and *EDS1*). Alongside, JA-associated genes, such as *PDF1.2* and *VSP2* were down-regulated (von Saint Paul et al., 2011). These data seem in contrast with the results obtained in this work, where we described an up-

regulation during *P.cucumerina* infection. However, the pathogen may inhibit the induction of genes involved in JA-defense response, as those involved in response to necrotrophic pathogens, and up-regulate repressor of this signaling pathway.

On the other side, *GLP5* encodes for a component of the plasmodesmata protein complex (Ham et al., 2012). *GLP5* is mainly expressed in roots and furthermore regulates primary root growth (Ham et al., 2012). The over-expression of GFP-tagged *GLP5* and its homolog *PDGLP2* reduced meristem size, causing thus altered root meristem and growth, suggesting a role of these proteins in the allocation of resources in roots (Ham et al., 2012). *GLP5* has also been associated with Fe-deficiency tolerance, as *GLP5* transcripts significantly up-regulated in the 0-Fe medium compared to Basal medium in Arabidopsis roots (Zargar et al., 2015). These roles may be important during salt stress and submergence, in both leaves and roots, but interestingly also in biotic stress. Other *GLP* family members in other plant species are express during different stresses, both biotic and abiotic (Dunwell et al., 2008). Therefore, GLP5 may control the allocation of resources in the stressful situations we applied in this work.

The induction of genes involved in cell wall remodeling, as *KOR2* and *PME17*, is not surprising in plants challenged with the stresses applied in this work. One of the responses to submergence is indeed an accelerated elongation growth to rise above the water line, and this movement is associated with changes in the cell wall composition and a reduction in cell wall rigidity (Lee et al., 2011). An altered cell wall architecture has also been found in *cpr5-2* and in the other *CPR5* recessive allele *cpr5-1* (Brininstool et al., 2008). Salt stress also provokes modification in the cell wall composition, particularly increasing the apposition of lignin (Shafi et al., 2015). Changes in cell wall composition are also important in the response to pathogens, as cell wall represents the first cellular barrier a pathogen has to face on (Bellincampi et al., 2014).

KOR2 codes for a member of the membrane-anchored endo-1,4-β-D-glucanases. These glucanases hydrolyze β-1,4-glucosidic linkages, playing thus a role in the cell wall biosynthesis during cell wall assembly (Brummell et al., 1997; Mølhøj et al., 2001). *KOR2::gusA* transgenic plants showed that plants accumulate KOR2 mainly in young developing root hairs and not in old root hairs, suggesting a role in early root hair development via cell wall modifications (Mølhøj et al., 2001). These data may explain the induction of *KOR2* during salt stress, as an increase in root hairs represents a typical response of plants after NaCl solution application (Arif et al., 2019).

PME17 is a member of the *group 2 Pectin methyl esterases* (*PMEs*) gene family, expressed mainly in primary roots, old leaves and stems (Sénéchal et al., 2014). PMEs catalyze the de-methyl esterification of homogalacturonans, the main components of the plant cell wall (Pelloux et al., 2007). PMEs inhibitor (PMEIs) proteins control the apoplastic activity of PMEs by post-translational modifications (Juge, 2006). Indeed, the degree of methyl esterification of homogalacturonans, mediated by PMEs-PMEIs regulation, plays a central role in different physiological processes, including plant-pathogen interaction (Hewezi et al., 2008; Osorio et al., 2008; Raiola et al., 2011). A high degree of methyl esterification protects pectins from microbial polygalacturonases and pectate lyases (Arancibia and Motsenbocker, 2006). Hence, it is not surprising to find *PME17* among the genes up-regulated during different abiotic and biotic stresses and in *cpr5-2*. Another member of the PME family, *PME31*, is also highly expressed during salt stress (Yan et al., 2018). Maybe, *PME17* could behave as its homolog and may regulate gene expression and salt stress tolerance. MYB transcription factor regulates the expression of *PMEIs* and indirectly of *PMEs*. For example, the transcription factor MYB46, which is involved in the regulation of secondary cell wall biosynthesis, mediates the expression of *PMEI* genes and regulates the susceptibility towards *B. cinerea* in Arabidopsis (Zhong et al., 2007; Ramírez et al., 2011). Interestingly, in our work, we found co-expressed both *PME17* and *MYB45*. Possibly, this transcription factor may regulate the expression of *PME17*.

According to publicly available data, most of the up-regulated uncharacterized genes accumulate in BABA-accumulating organs, such as senescent leaves, flowers and particularly seeds (**Fig. 3-4A**, **S3-4A** and **S3-5A**) (Balmer et al., 2019), where only *MYB45* showed no accumulation (**Fig. S3-3A**). However, salt stress induced all genes in roots but not in leaves, in contrast to our data that show a leaf-up-regulation pattern (**Fig. S3-3B**, S**3-4B, S3-5B**). It would be thus interesting to look at gene expression in roots after salt stress to confirm these data. With the exclusion of AT1G30700, up-regulated genes showed no induction after hypoxia (**Fig. 3-4C** and **S3-5C**), with also a down-regulation of *AtMYB45* (**Fig. S3-3C**). Possibly, other submergence-associated stresses up-regulate these genes. *Pst* avrRpm1 induces the up-regulated genes found with RNA-seq, with AT4G11170 transcripts with high expression already 2 hpt (**Fig. 3-4D**, **S3-3D, S3-4D**, **S3-5D**). These data looked similar to our data after *Pst* AvrRpt2 infection. Finally, all the up-regulated genes showed induction after inoculation with the necrotrophic pathogen *B. cinerea*, similarly as we found with *P. cucumerina* (**Fig. 3-4E**, **S3-3E, S3-4E**, **S3-5E**). Nevertheless, few public-available data exist for the down-regulated gene AT5G38865, excluding a high induction presumed in stamen (**Fig. S3-6**).

However, the main goal of this work was to find genes regulating both biosynthesis and accumulation of BABA in plants. To check if the up-regulated genes found with RNA-seq are involved in BABA metabolism, we decided to look at BABA concentration after salt stress in T-DNA insertional lines of the up-regulated genes. Most of the insertions place in the coding region, while others, such as in *glp5-1* and *kti4.1* in the promoter. Unfortunately, all the lines used showed BABA levels compared to wild-type 48 hpt after NaCl (**Fig. 3-5**). We used this harvesting point as it represents a peak in concentration for BABA during salt stress. Therefore, the first explanation for this wild-type accumulation of BABA in T-DNA lines may be a not correct choice for the time frame for RNA extraction. We arbitrary selected the harvesting point regulating it on the time of BABA peak in concentration reported in the literature, considering that the transcripts may be highly expressed.

Furthermore, we assumed that BABA-related genes transcription follows the "signal delay" model, but these genes may be transcribed by following other models. The "translation on-demand" model permits to increase translation without inducing mRNA concentrations and to activate thus protein biosynthesis rapidly after a stimulus (Liu et al., 2016). This model ensures proteins quickly available (Liu et al., 2016). This may be the case of the BABA biosynthetic gene that could be rapidly mobilized for the synthesis of a possible signaling molecule. Besides, utilizing this model, plants avoid waste of energy and resources, particularly nitrogen, for the synthesis of new RNA. However, *cpr5-2* is impaired in nucleocytoplasmic transport of transcripts. Therefore, the transcription of BABA-related genes seems to better fit with the "signal delay" model than with "translation on-demand".

Finally, the pattern of BABA accumulation during salt stress observed may depend on the physiological roles played by the found genes. The responses regulated by *MYB45* and *HSFB1* transcription may not have a relation with BABA at all, but to control other players of plant response to stresses. Mutation in *GLP5* may disrupt the allocation of resources during stress and alter the biosynthetic process of BABA. Nevertheless, the mutation in the promoter of *GLP5* could not be strong enough to interfere with the correct expression of the gene. *KTI1*, *KOR2,* and *PME17* represent respectively a protease inhibitor and cell wall remodeling genes. Consequently, the wild-type BABA accumulation may not change as the biosynthetic and the regulatory pathway keep unaltered. Nevertheless, the lines used in this work displayed a wild-type BABA induction after salt stress. For *MYB45* and *AT1G13830,* the data come from homozygous lines, while for the other two, we sowed a large population, but no individuals have altered induced-BABA concentrations. We could hypothesize that the lines we used do

not alter the biochemistry of the protein encoded or do not knocked-down the transcript. Unfortunately, we cannot reach this objective with the experiments performed. Therefore, new experiments have to be set-up for searching for new genes involved in BABA metabolism in Arabidopsis.

3.5. References

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3.6. Supplementary material

Gene name	Gene locus	T-DNA insertion name	Type	Back-ground	Mutant name
GLP5	AT1G09560	SALK_094426C	Homozygous	Col-0	*glp5-1*
AT1G13830	AT1G13830	SALK_110849	Segregating	Col-0	
AT1G30700	AT1G30700	SALK_117810	Segregating	Col-0	
KOR2	AT1G65610	SALK_006954C	Homozygous	Col-0	*kor2-1*
KTI1	AT1G73260	SALK_131716C	Homozygous	Col-0	*kti4.1* (Arnaiz et al., 2018)
PME17	AT2G45220	SALK_059908C	Homozygous	Col-0	*pme17-2* (Sénéchal et al., 2014)
UGT76B1	AT3G11340	SAIL_1171A11	Homozygous	Col-0	*utg76b1-1* (von Saint Paul et al., 2011)
AtMYB45	AT3G48920	SALK_037556C	Homozygous	Col-0	*myb45-1*
AT4G11170	AT4G11170	SALK_112198	Segregating	Col-0	
HSFB1	AT4G36990	SALK_104713C	Homozygous	Col-0	*hsfb1-* (Pick et al., 2012)

Table S3-1: Description of the genes and T-DNA lines used for this project.

Sample ID	Sample name	Replicate	Description
01	Col0	01	Total RNA from untreated 5 weeks-old Arabidopsis Col-0 plants used as control for NaCl, submergence and *cpr5-2* samples
02	Col0	02	
03	Col0	03	
04	NaCl	01	Total RNA from 5 weeks-old Arabidopsis Col-0 plants treated with 200 mM NaCl solution and harvested and processed at 18 hpt
05	NaCl	02	
06	NaCl	03	
07	Sub	01	Total RNA from 5 weeks-old Arabidopsis Col-0 plants submerged for 24 hours until run-off.
08	Sub	02	
09	Sub	03	
10	cpr5	01	Total RNA from 5 weeks-old untreated *cpr5-2* mutants in Col-0 background.
11	cpr5	02	
12	cpr5	03	
13	MgCl2	01	Total RNA from 5 weeks old Arabidopsis Col-0 plants treated infiltrating leaves with a solution of 10 mM $MgCl_2$ and harvested 18 hpt, controls for AvrRpt2 samples
14	MgCl2	02	
15	MgCl2	03	
16	AvrRpt2	01	Total RNA from 5 weeks old Arabidopsis Col-0 plants treated infiltrating leaves with a suspension of 10^7 cfu/mL of AvrRpt2 in 10 mM $MgCl_2$ and harvested and processed 18 hpt
17	AvrRpt2	02	
18	AvrRpt2	03	
19	PDB/2	01	Total RNA from 5 weeks old Arabidopsis Col-0 plants sprayed with PDB/2 and harvested and processed and processed at 36 hpt, controls for *P. cucumerina* samples
20	PDB/2	02	
21	PDB/2	03	
22	*Pcuc*	01	Total RNA from 5 weeks old Arabidopsis Col-0 plants sprayed with a suspension of *P. cucumerina* (5×10^6 spores mL^{-1} in PDB/2) and harvested and processed and processed at 36 hpt
23	*Pcuc*	02	
24	*Pcuc*	03	

Table S3-2: Description of the RNA libraries used for RNA-seq. Each library has been associated with a sample ID and a sample name, representative of the type of treatment applied, or of the type plant of the RNA extraction experiment. In the last column, a brief description of the RNA material composing the RNA library.

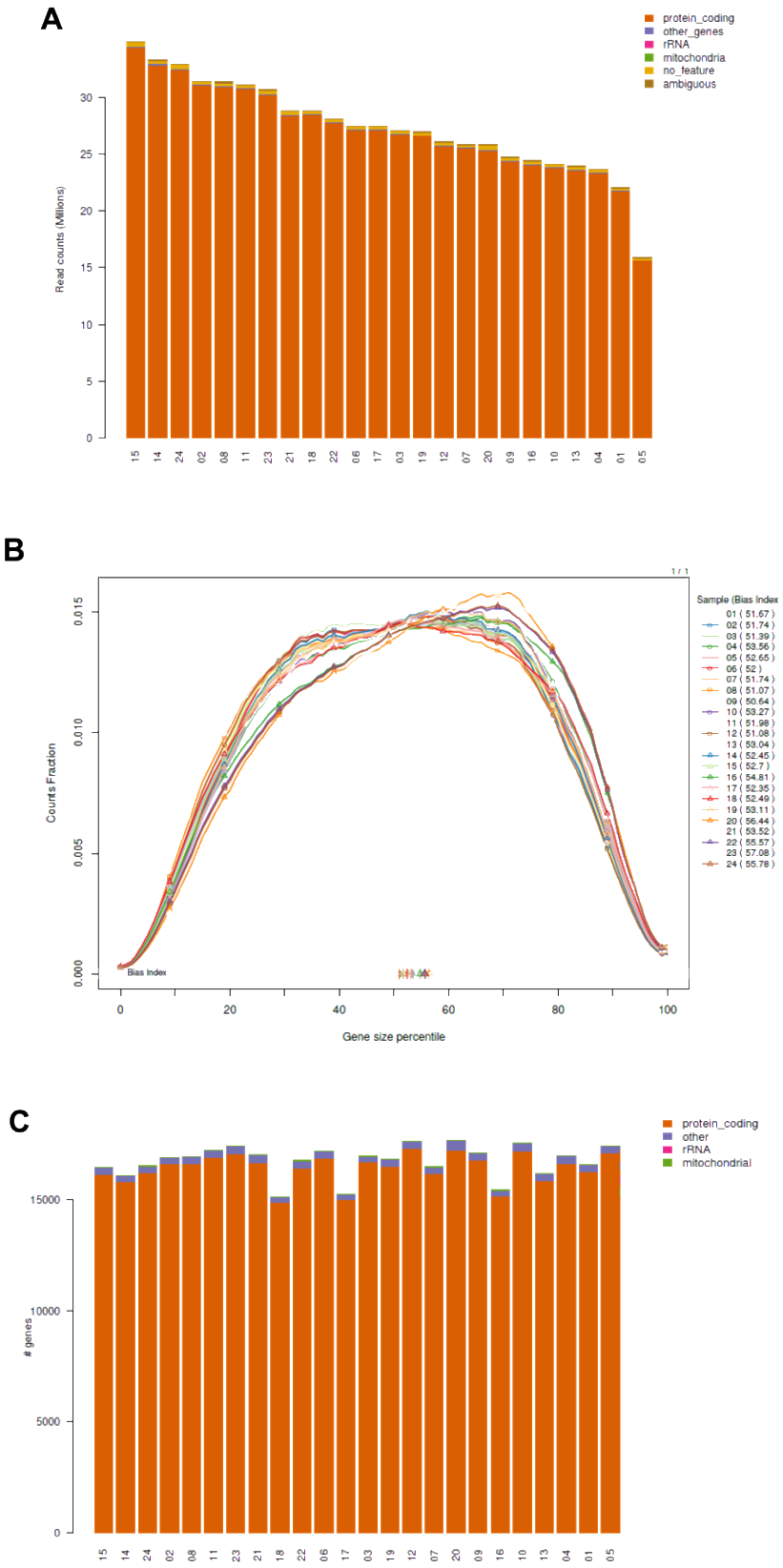

Fig. S3-1: RNA-seq quality control report. A: Number of read counts per gene locus summarized with HTSEQ-count using *Arabidopsis thaliana* gene annotation TAIR10.39. Genes categories are divided according to their characteristics and marked with a different color. **B:** RSeQC gene body coverage of protein-coding genes in each sample, marked by a different color. **C:** Expressed genes reads per kilobase per million (RPKM ≥ 1) per each sample divided according to their characteristics.

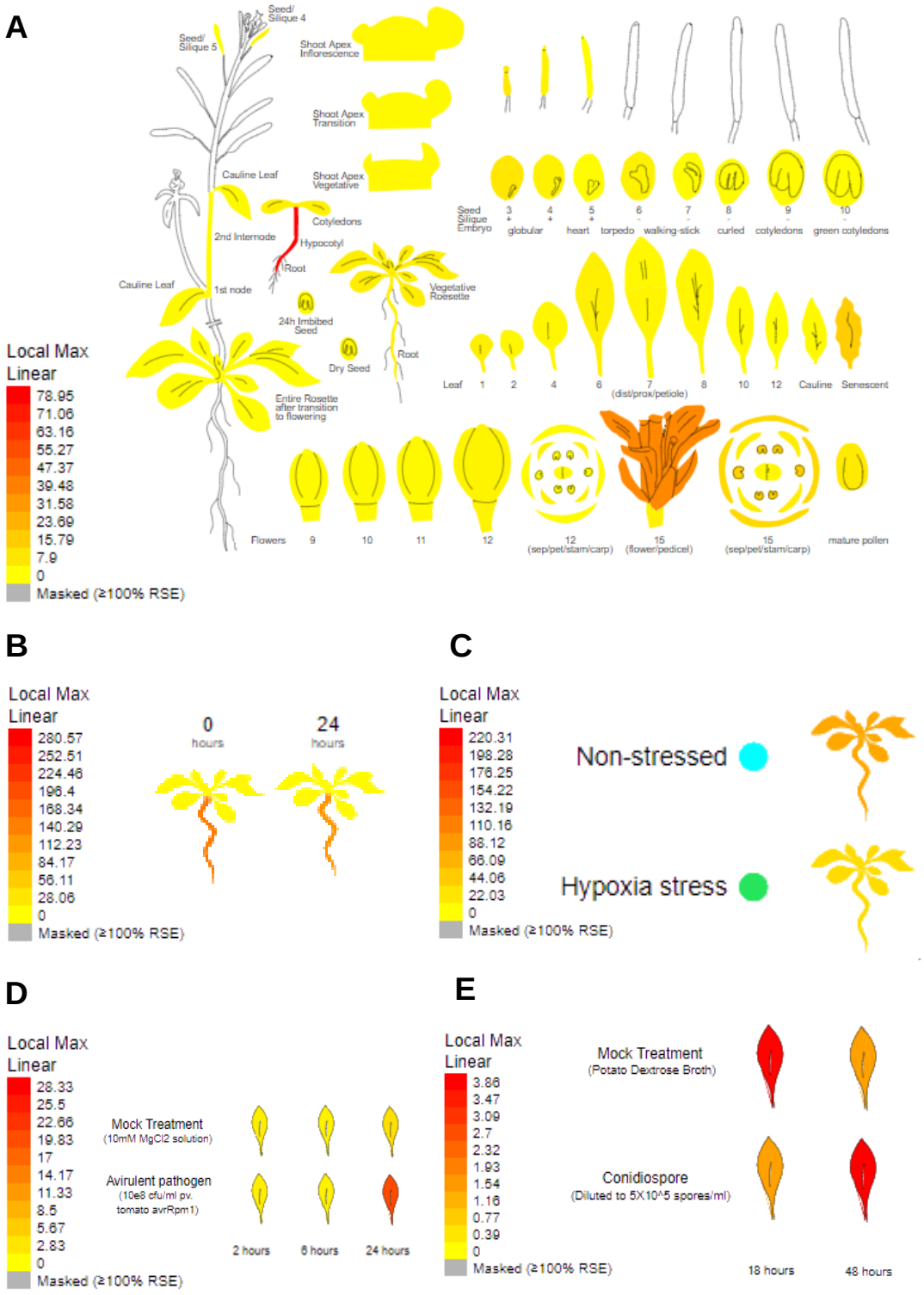

Fig. S3-2: Pattern of expression of *AtMYB45*, according to efp Browser. A: Anatomical map of Arabidopsis generated with the AtGenExpress eFP (bar.utoronto.ca/eplant) and modified for this work showing the pattern of expression of *AtMYB45*. **B:** Pattern of expression of *AtMYB45* in 18-day-old wild-type Col-0 plants treated with 150 mM NaCl and harvested at time 0 and 24 hpt. **C:** Pattern of expression of *AtMYB45* in 7-day-old Arabidopsis Ler plants cultivated on MS agar plates and grown for 12 hours with argon gas until run-off. **D:** Pattern of expression of *AtMYB45* in leaves of 5-week-old Arabidopsis Col-0 plants infiltrated with 10 mM $MgCl_2$ and with a suspension of 10^8 cfu/mL of *Pst* AvrRpm1. **E:** Pattern of expression of *AtMYB45* in 4-week-old Arabidopsis Col-0 drop-inoculated with a 5 x 10^5 spores/mL *B. cinerea* suspension and with PDB/2 broth. Lower expression strength is expressed by the color yellow, while higher is expressed in red. Images were generated with the AtGenExpress eFP (bar.utoronto.ca/eplant).

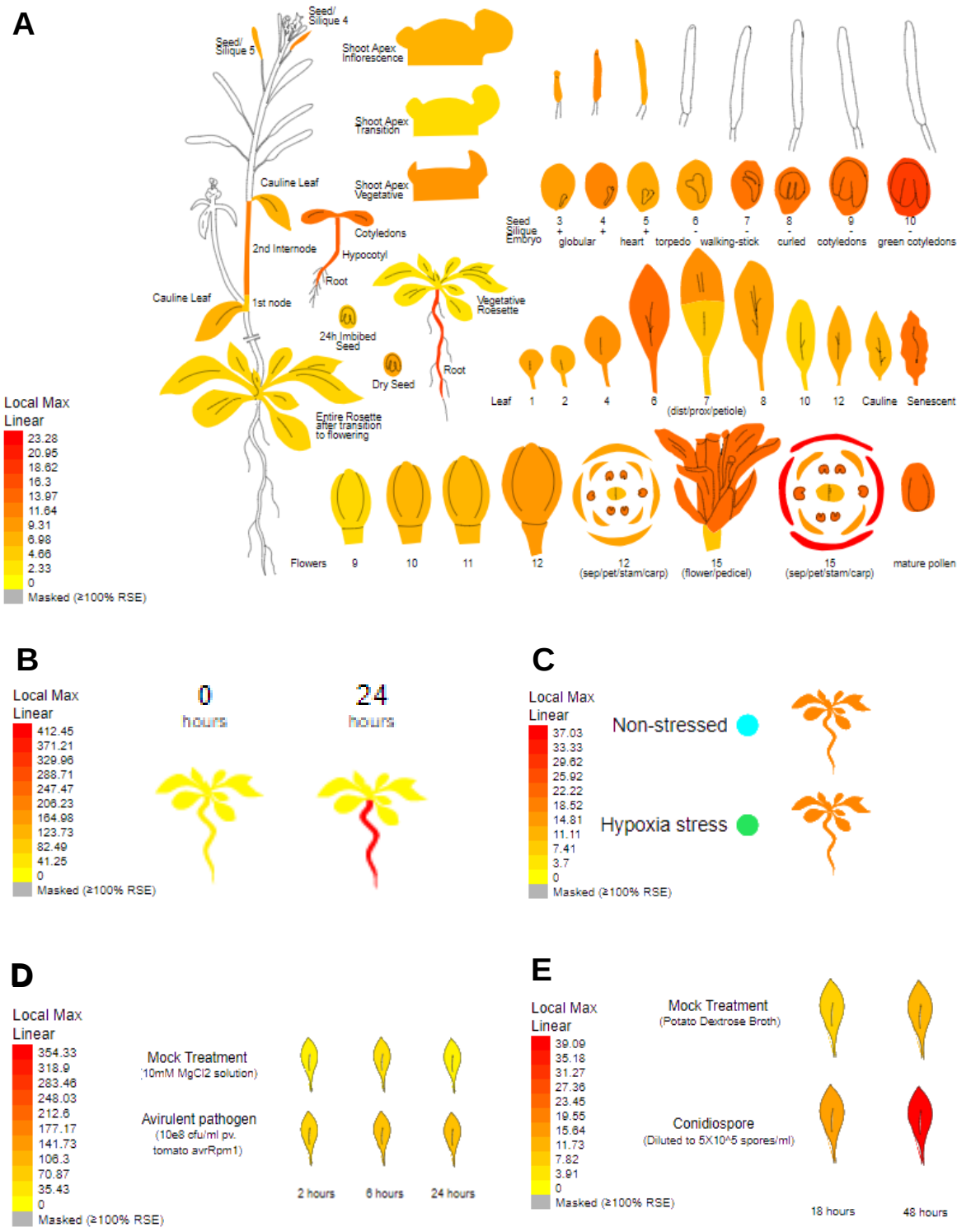

Fig. S3-3: Pattern of expression of AT4G11170, according to efp Browser. A: Anatomical map of Arabidopsis generated with the AtGenExpress eFP (bar.utoronto.ca/eplant) and modified for this work showing the pattern of expression of AT4G11170. **B:** Pattern of expression of AT4G11170 in 18-day-old wild-type Col-0 plants treated with 150 mM NaCl and harvested at time 0 and 24 hpt. **C:** Pattern of expression of AT4G11170 in 7-day-old Arabidopsis Ler plants cultivated on MS agar plates and grown for 12 hours with argon gas until run-off. **D:** Pattern of expression of AT4G11170 in leaves of 5-week-old Arabidopsis Col-0 plants infiltrated with 10 mM $MgCl_2$ and with a suspension of 10^8 cfu/mL of *Pst* AvrRpm1. **E:** Pattern of expression of AT4G11170 in 4-week-old Arabidopsis Col-0 drop-inoculated with a 5 x 10^5 spores/mL *B. cinerea* suspension and with PDB/2 broth. Lower expression strength is expressed by the color yellow, while higher is expressed in red. Images were generated with the AtGenExpress eFP (bar.utoronto.ca/eplant).

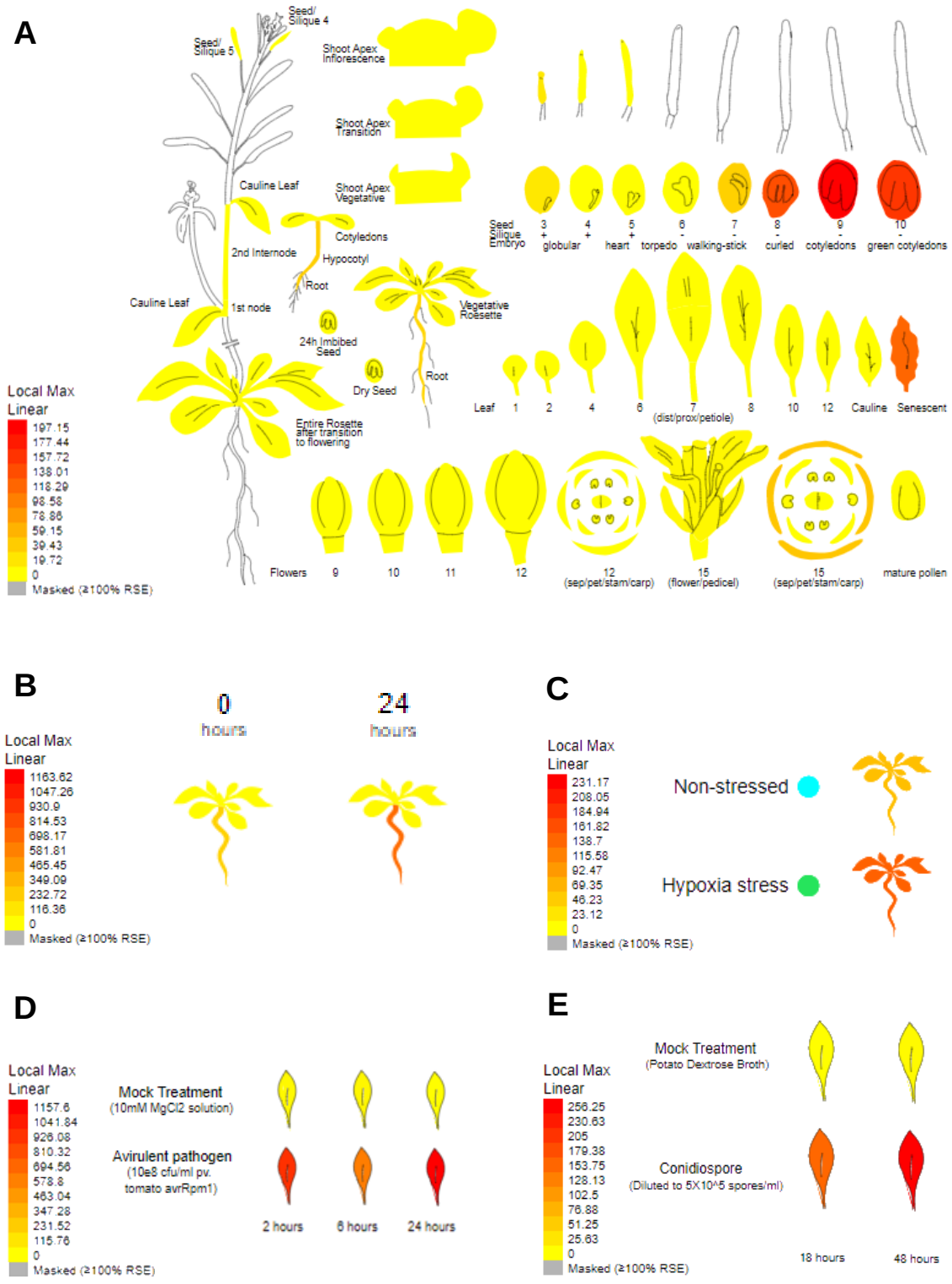

Fig. S3-4: Pattern of expression of AT1G30700, according to efp Browser. A: Anatomical map of Arabidopsis generated with the AtGenExpress eFP (bar.utoronto.ca/eplant) and modified for this work showing the pattern of expression of AT1G30700. **B:** Pattern of expression of AT1G30700 in 18-day-old wild-type Col-0 plants treated with 150 mM NaCl and harvested at time 0 and 24 hpt. **C:** Pattern of expression of AT1G30700 in 7-day-old Arabidopsis Ler plants cultivated on MS agar plates and grown for 12 hours with argon gas until run-off. **D:** Pattern of expression of AT1G30700 in leaves of 5-week-old Arabidopsis Col-0 plants infiltrated with 10 mM $MgCl_2$ and with a suspension of 10^8 cfu/mL of *Pst* AvrRpm1. **E:** Pattern of expression of AT1G30700 in 4 week old Arabidopsis Col-0 drop-inoculated with a 5 x 10^5 spores/mL *B. cinerea* suspension and with PDB/2 broth. Lower expression strength is expressed by the color yellow, while higher is expressed in red. Images were generated with the AtGenExpress eFP (bar.utoronto.ca/eplant).

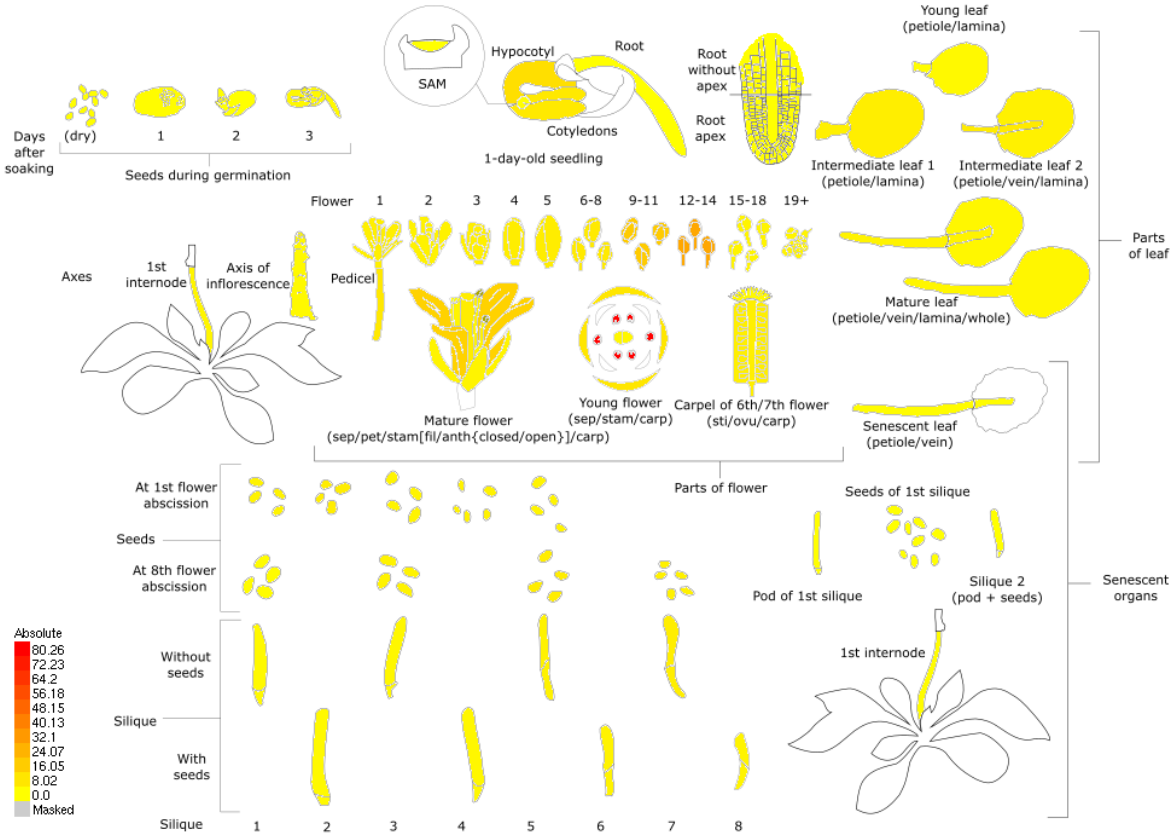

Fig. S3-5: Pattern of expression of AT5G38865, according to Klepikova efp. Map of *Arabidopsis thaliana* developmental transcriptome based on RNA-seq profiling from Klepikova et al. (2016) concerning AT5G38865. Lower expression strength is expressed by the color yellow, while higher is expressed in red. Images were generated with the AtGenExpress eFP (bar.utoronto.ca/eplant).

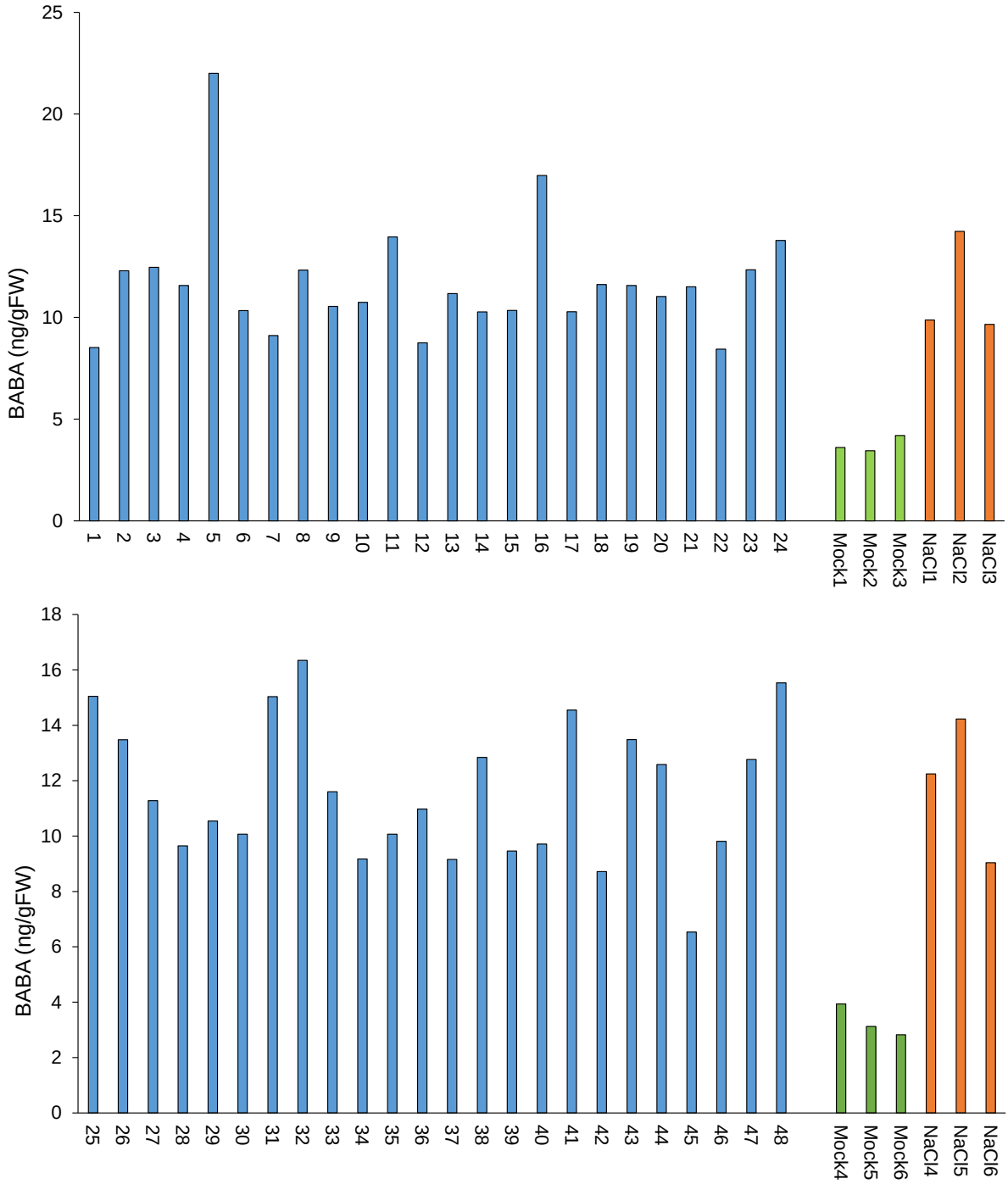

Fig. S3-6: BABA concentrations in a segregating population of the line SALK_110849 after salt stress. Blue bars represent BABA concentrations in the individual population of SALK_110849 treated with a soil-drench 200 mM NaCl solution. The mock-treated wild-type Col-0 individuals are marked in green. Orange bars represent NaCl-treated wild-type Col-0 plants.

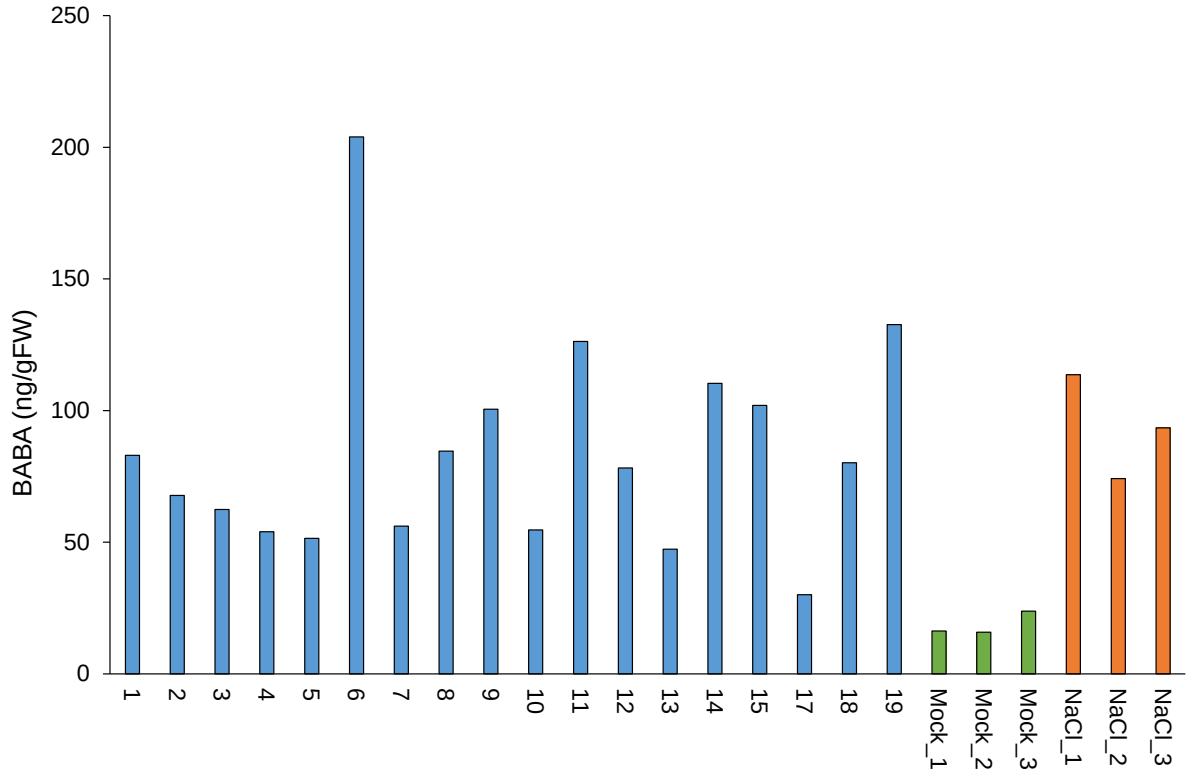

Fig. S3-7: BABA concentrations in a segregating population of the line SALK_112198 after salt stress. Blue bars represent BABA concentrations in the individual population of SALK_112198 treated with a soil-drench 200 mM NaCl solution. The mock-treated wild-type Col-0 individuals are marked in green. Orange bars represent NaCl-treated wild-type Col-0 plants.

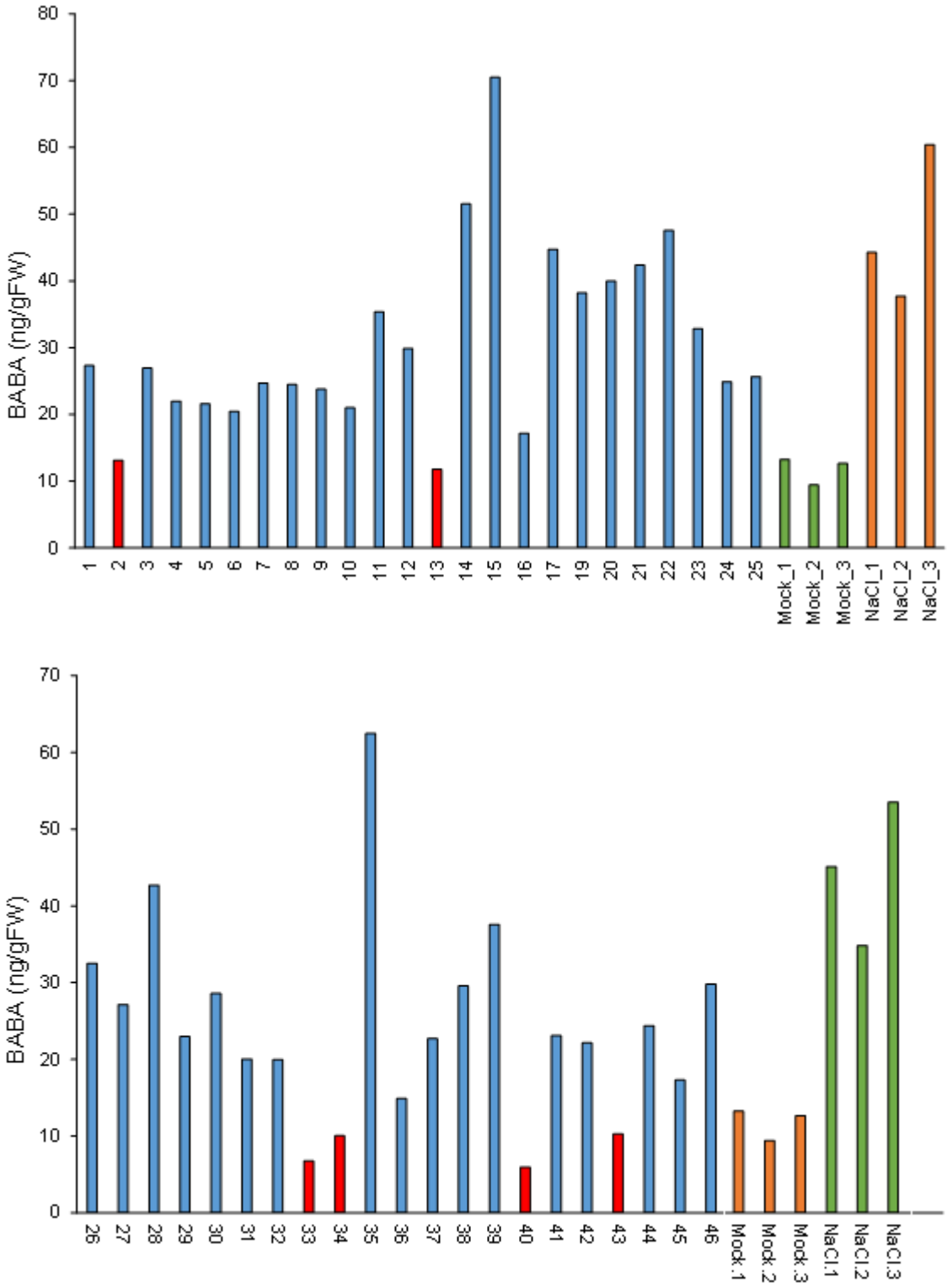

Fig. S3-8 : BABA concentrations in a segregating population of the line SALK_110849 after salt stress. Blue bars represent BABA concentrations in the individual population of SALK_110849 treated with a soil-drench 200 mM NaCl solution. Red bars represent individuals with lower or similar BABA concentrations compared to the three mock-treated wild-type Col-0 individuals, marked in green. Orange bars represent NaCl-treated wild-type Col-0 plants.

Supplementary file: Ftest_abioticstresses,AvRpt2andPcucumerina.xls

4. Dissecting the relation between the plant metabolite BABA and phytohormones, with emphasis on ABA-signaling.

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Hormones play vital roles in plant physiology, from development to stress response. The priming agent β-aminobutyric acid (BABA) activates different hormonal pathways for the onset of resistance repertoire in plants. Plant synthesize BABA and accumulate it after stressful conditions. However, the hormonal regulation of BABA accumulation was not known. Therefore, we applied phytohormones solutions to Arabidopsis plants, and we analysed theBABA concentrations. Only abscisic acid (ABA) was able to increase BABA levels in plants, with the bioactive form (+)-ABA showing a more robust induction. The three *SnrK2s* genes are key regulators of ABA signaling in Arabidopsis. To confirm that ABA-dependent BABA induction is under the control of ABA signaling, we treated the ABA-signaling mutants *snrk2.2 snrk2.3* and *snrk2.6* with an (+)-ABA solution. The mutants showed wild-type BABA concentrations, suggesting a possible compensation effect among the three genes.

4.1. Introduction

Plant hormones are defined as low concentrated signaling molecules, whose changes in concentrations after an external or physiological stimulus mediate the response to different physiological processes (Buchanan et al., 2015). Various molecules in plants act as plant hormones. For a first example, auxins regulate root and shoot architecture, fruit and flower development, cell elongation and tissue differentiation. The main auxin synthesized in higher plants is indol-3-acetic acid (IAA) (Wang and Fu, 2011). Auxins were first characterized as regulator of tropism and their presence was already inferred by Charles and Francis Darwin (Darwin, 1897; Leyser, 2010). Then, giberellins (GAs) are diterpenoid carboxylic acids present in plants and as well as in fungi and bacteria (Hedden and Thomas, 2012). GAs stimulate cell elongation and consequently organ growth, promote seed dormancy and germination, vegetative and reproductive development, and play also a positive role in fertility (Hedden and Thomas, 2012). GA_1, GA_3 and GA_4 are the active hormonal forms of GAs, and among them, GA_3, also known as gibberellic acid which is the commercialized form (Hedden and Thomas, 2012). Another example, cytokinins regulate different aspects of plant growth, such as by promoting cell division and inhibiting senescence. Cytokinins are a vast family of plants hormones; among them, Zeatin is the first cytokinin discovered in plants (Letham, 1963). Plants can also perceive the synthetic cytokinin Kinetin, which is used to alleviate salt stress effects in maize and in other species (Miller et al., 1955; Alla et al., 2002; Hamayun et al., 2015). In the study of plant-pathogen interaction, the most characterized hormones are SA, JA and ET, although they also play a role in abiotic stress response (Fujita et al., 2006; Robert-Seilaniantz et al., 2011). SA is a phenolic plant hormone able to induce the HR and the expression of the antimicrobial PR proteins in local infected tissues (Cameron et al., 1999; Alvarez, 2000). SA is also the main signaling molecule in the activation of SAR and in the response to biotrophic pathogens (Fu and Dong, 2013). JA, by contrast, activates the defenses against herbivores and necrotrophic pathogens, by activating the defense response in systemic tissues, also with the help of its volatile methylated form (MeJA) (Seo et al., 2001; Pieterse et al., 2014). Along with JA, ET is a volatile hormone involved in the response to necrotrophic pathogens and herbivores (Baldwin et al., 1997), playing also an important role in senescence and fruit ripening and abscission (Payton et al., 1996).

ABA is a plant hormone involved in various physiological processes, spanning from abiotic stress tolerance to seed dormancy and plays a role in plant defense as well, mediating stomatal closure to avoid pathogen penetration for example (Finkelstein, 2013). ABA signaling functions via a complex network composed of several players, which interact with each other to gather the information, integrate the signal and finally output it (**Fig. 4-1**) (Hirayama and Umezawa, 2010; Nakashima and Yamaguchi-Shinozaki, 2013). The signal starts when ABA is perceived and bound by one of the fourteen members of the cytoplasmic Pyrabactin Resistance/Pyrabactin 1-Like or Regulatory Components of ABA Receptor (PYR/PYL/RCAR) protein family (Ma et al., 2009). PYR/PYL/RCARs interact in turn with the nine members of the Clade A Protein Phosphatase 2C (PP2C) protein family which work as negative regulators of ABA signaling in plants (Schweighofer et al., 2004; Xue et al., 2008; Ma et al., 2009; Park et al., 2009). In fact, PP2CAs inactivate by dephosphorylating the three members of class III SNF1-Related Kinases 2 (SnRK2) (**Fig. 4-1**) (Gosti et al., 1999; Umezawa et al., 2009; Vlad et al., 2009). By contrast, the binding of ABA with PYR/PYL/RCARs inactivates PP2CAs and releases the SnRK2s (Park et al., 2009; Santiago et al., 2009). SnRK2s are the key regulators of ABA signaling, working as activator kinases for downstream transcription factors, belonging to the AREB/ABF bzip family (**Fig. 4-1**) (Kobayashi et al., 2005; Furihata et al., 2006). Successively, phosphorylated AREB/ABF transcription factors bind the ABA-responsive (ABRE) cis-elements, or in combination with coupling elements (CE), activating thus the transcription of ABA-responsive genes (Nakashima and Yamaguchi-Shinozaki, 2013). Thirty-eight genes belong to the SnRK protein family, which is divided in turn into three subgroups (Hrabak et al., 2003). Ten genes belong to the subgroup 2, but only *SnRK2.2*, *SnRK2.3* and *SnRK2.6/Ost1* work in the ABA signaling (**Fig. 4-1**) (Mustilli et al., 2002; Hrabak et al., 2003; Fujii et al., 2007; Fujii and Zhu, 2009). *SnRK2.6/Ost1* is mainly expressed in guard cells and is involved in the ABA-dependent response to osmotic stress (Mustilli et al., 2002), while *SnRK2.2* and *SnRK2.3* play a role in the ABA-signaling during seedling growth and seed germination and dormancy (Fujii et al., 2007).

The priming-inducing agent BABA is a plant molecule constitutively present at low levels, that increase after biotic and abiotic stress (Thevenet et al., 2017). BABA molarity in plants is similar to those of other hormones (Baccelli and Mauch-Mani, 2017). Furthermore, BABA induction under different stresses was comparable to other phytohormonal dynamics (Baccelli and Mauch-Mani, 2017). These characteristics suggest that BABA may be a plant hormone, but more information in BABA biosynthesis and regulation is needed to confirm this hypothesis

(Baccelli and Mauch-Mani, 2017). However, application of BABA to Arabidopsis activates different phytohormone pathways to induce defense against pathogens and abiotic stress. In fact, BABA-IR against *Pst* DC3000 requires a functional SA signaling, as the Arabidopsis SA-signaling mutant *npr1-1* and the SA-deficient mutants *NahG* were impaired in expressing BABA-IR (Zimmerli et al., 2000). Furthermore, *PRs* transcripts expression during BABA-IR is also dependent on SA-signaling, as *B. cinerea* infected *NahG* and *npr1-1* mutant did not show BABA-primed *PR-1* induction (Zimmerli et al., 2001). Furthermore, BABA potentiates JA-dependent genes, such as *LOX-9* and *PR-4*, in *P. viticola* infected grapes (Hamiduzzaman et al., 2005). However, Arabidopsis JA-related mutants *jar1* expressed a wild-type BABA-IR during *Pst* DC3000 infection (Zimmerli et al., 2000). Similar results were obtained in *Pst* DC3000 infected ET-receptor mutant *etr1* plants (Zimmerli et al., 2000). However, BABA primes *ETR1* expression, a negative regulator of ET-signaling, during osmotic stress in potato, suggesting a negative role of BABA in this hormonal pathway (Sós-Hegedűs et al., 2014).

A functional ABA signaling is required for the BABA-IR against penetrating fungi and oomycetes (Baccelli and Mauch-Mani, 2016). In fact, the ABA-insensitive mutant *abi4-1* is impaired in BABA-enhanced callose deposition during *P. cucumerina* infection (Ton and Mauch-Mani, 2004). ABA-signaling is also important in BABA-priming against osmotic stress, considering that BABA-treated *abi4-1* shows a water loss similar to mock-treated plants after days of dehydration (Jakab et al., 2005). Despite the importance during BABA-IR, little is known about the relation between ABA and plant-produced BABA.

To look whether plant hormones regulate BABA concentrations in Arabidopsis, we treated Col-0 plants with different phytohormone solutions. Interestingly, we found that ABA increased the concentrations of BABA when applied as soil-drench. The (+)-ABA bioactive form induced BABA when sprayed and drenched in soil. The ABA-signaling mutants *SnRK2s* did not differ from the wild type in inducing BABA following ABA treatment. We propose that redundancy in this protein family is responsible for the wild-type BABA induction in the *SnRK2s* mutants.

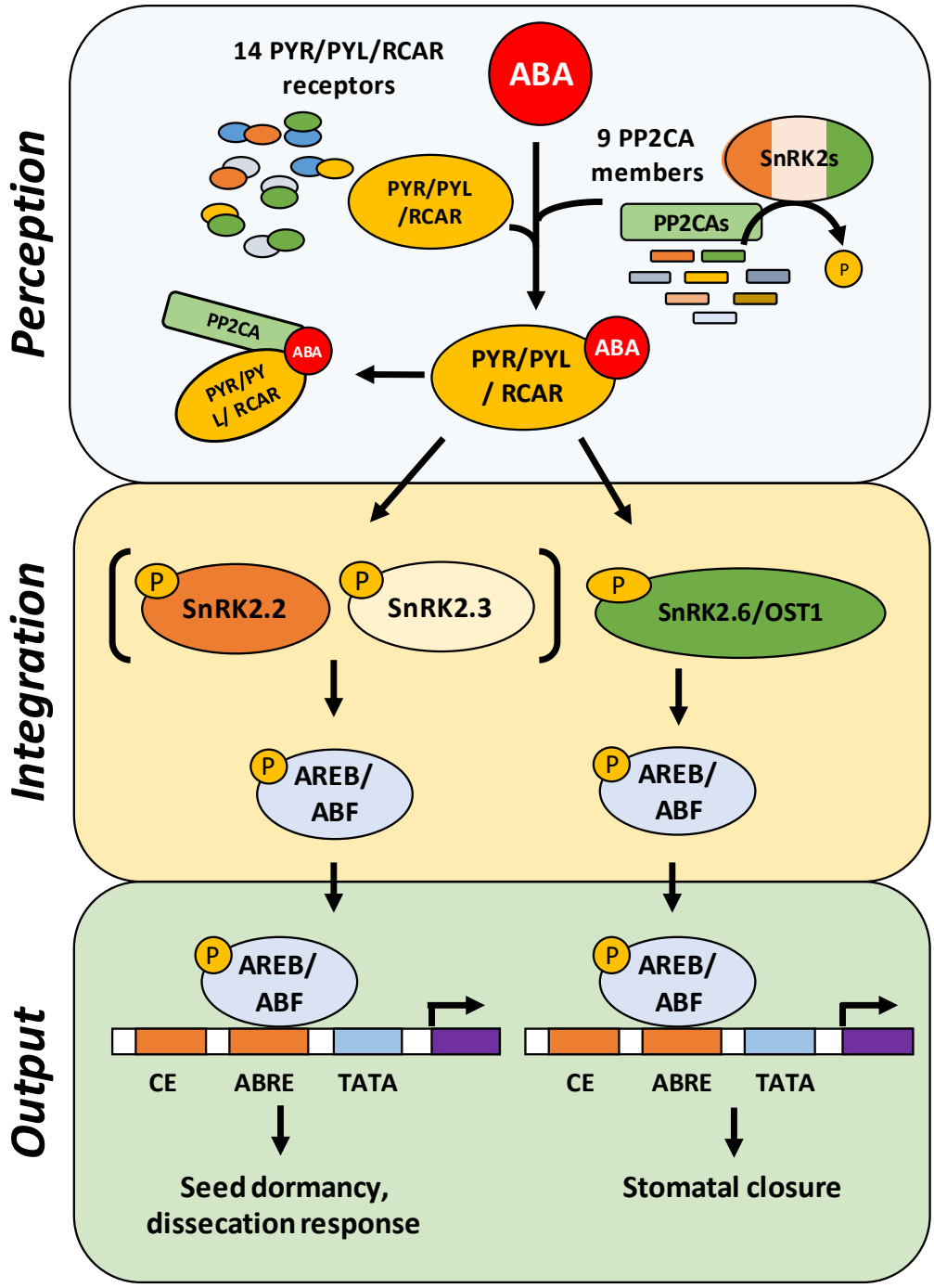

Fig. 4-1: ABA-signaling network in plants. ABA perception starts when a member of the PYR/PYL/RCAR receptor family (composed of fourteen genes) binds the hormone. The perception of ABA and PYR/PYL/RCARs releases the SnRK2 kinases, by binding the PP2CA negative regulator of ABA-signaling. In unchallenged conditions, the nine members of the PP2CA protein family dephosphorylate the three SnRK2s implied in ABA-signaling, inactivating their kinase activity. Once released, SnRK2 kinases integrate the signal, phosphorylating the AREB/ABF transcription factors, that in turn bind ABRE and CE cis element on promoters of ABA-activated genes. The three kinases control different aspects of ABA signaling. SnRK2.2 and SnRK2.3 activate the ABA signaling involved in seed dormancy and desiccation response, while SnRK2.6/OST1 is mainly involved in stomatal closure. Modified from Hirayama and Umezawa (2010) and Nakashima and Yamaguchi-Shinozaki (2013).

4.2. Material and methods

4.2.1. Plant material and growth condition

Seeds of *Arabidopsis thaliana* plants accession Columbia-0 (Col-0) were multiplied from a stock purchased at Lehle Seeds, Round Rock, TX, USA. Seeds of *snrk2.2 snrk2.3* (*GABI-Kat 807G04/ SALK_107315*) were kindly supplied by L. Lopez-Molina (Department of Plant Biology, University of Geneva, Geneva, Switzerland). Seeds of *snrk2.2/snrk2.3/snrk2.6* triple mutant (*GABI-Kat 807G04/ SALK_107315/SALK_008068*) were shared with P. Wang (Shanghai center for plant stress biology, Shanghai, P.R. China). Seeds of homozygous *snrk2.6/ost1-3* (*SALK_008068*) were purchased at NASC.

Plants were sown on hydrated Jiffy-7 peat pellets and stratified at 4°C for 3 days before being transferred in growth cabinets at 21°C day and 18°C night, at short day condition (8 h light at 100 µmol/m^2/s^{-1}) and 70% relative humidity (RH). The experiments were performed when plants reached the age of 5 weeks.

4.2.2. Preparation of chemical solutions and treatments

Methyl Jasmonate (MeJA) solution was prepared by diluting a defined volume of MeJA 95% solution (Sigma-Aldrich) in mqWater. The ET precursor ACC (Van de Poel and Van Der Straeten, 2014) solution was prepared by starting from ACC powder (Sigma-Aldrich) then diluted in mqWater. Powders of salicylic acid (SA), Giberellic acid (GA_3), indol-3-acetic acid (IAA), racemic ABA and (+)-ABA were purchased at Sigma-Aldrich. Stock solutions were prepared by dissolving the different powders in 1 mL of ethanol and then diluted in mqWater. Kinetin powder (Sigma-Aldrich) was first dissolved in 1 mL of 1N NaOH solution and then diluted in mqWater.

For spray inoculation of plants at a concentration of 100 µM containing 0.01% Silvett-L 77 (Thermo Fischer Scientific) was used. Soil drenching 10 was performed with a 300 µM solution to reach a final concentration of 100 µM in the soil. For each treatment, we used as control the same solvent as for the chemical. The aerial part of treated and control plants was harvested and flash-frozen in liquid nitrogen and stored at -80 °C.

4.2.3. Quantification of BABA concentrations

BABA extraction and quantification was performed according to Balmer et al. (2019) using Acquity UPLC system (Waters, Milford, MA, USA) coupled to a TQ-S triple quadrupole (Waters, Milford, MA, USA) in electrospray positive mode. The BABA quantification was performed by the Neuchatel Institute of Analytical Chemistry from the University of Neuchâtel.

4.2.4. Statistical analysis

BABA concentrations were analyzed using Student's t-test. Statistical analysis was performed using Microsoft Excel.

4.3. Results

4.3.1. Effect of treatment with different phytohormones on BABA concentrations in Arabidopsis

To determine the effect of the application of phytohormones on BABA concentrations in plants, we applied different phytohormone solutions by soil-drench or spraying on 5-week-old Arabidopsis Col-0 plants. We selected (±)-ABA, MeJA, SA, GA_3, ACC, Kinetine, and IAA.

Plant material was collected 48 hours and 72 hours post-treatment (hpt) and processed to extract and detect BABA concentrations. With the exception of ABA, none of the other phytohormones applied changed BABA concentrations compared to the control in both spray (**Fig. S4-1**, **S4-2** and **S4-3**) and soil-drench treatments (**Fig. 4-2A** and **S4-2**). Interestingly, soil-drench with the racemic ABA solution led to a significant increase in BABA concentrations 48 and 72 hpt (**Fig. 4-2B**), while ABA spray treatment did not induce BABA significantly at both 48 and 72 hpt (**Fig. S4-1**).

4.3.2. The bioactive (+)-ABA enantiomer induces BABA in both spray and soil-drench treatment

To determine whether the ABA-dependent BABA induction was due to the bioactive form of ABA, (+)-ABA (Cutler et al., 2010), we applied a solution of 100 µM final concentration of

(+)-ABA as soil-drench and spray respectively, and harvested plant aerial material 48 and 72 hpt.

(+)-ABA increased BABA concentrations 48 and 72 hpt in soil-drench treated plants (**Fig. S4-4**) and in contrast to racemic ABA also in sprayed plants (**Fig. S4-4**). Spraying a series of (+)-ABA solutions at increasing concentrations (0 to 100 µM), BABA levels increased starting at 25 µM 72 hpt in a concentration-dependent manner (**Fig. 4-3A**). Interestingly, the stronger the (+)-ABA-dependent phenotype is on the plant, the higher were the concentrations of BABA detected (**Fig. 4-3B**). These preliminary data suggest that the bioactive form of ABA is responsible for the induction of BABA in both spray and soil-drench treatments, but further experiments with the inactive form (-)-ABA have to be performed.

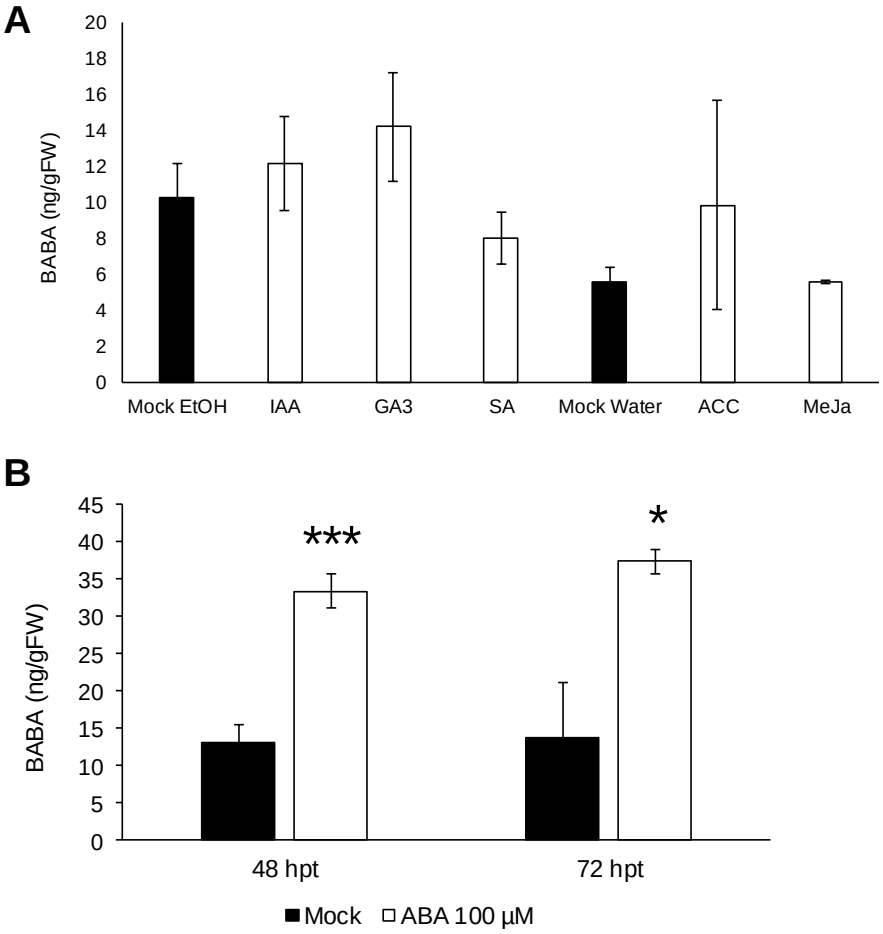

Fig. 4-2: Hormonal soil-drench treatment effect on BABA concentrations in Arabidopsis Col-0 plants 48 hpt. A: 5-week-old Arabidopsis Col-0 plants were drenched with phytohormone solutions of 100 µM final concentration in the soil. Mock treated plants were treated drenching in soil solutions without the corresponding hormone. **B**: Racemic ABA soil-drenched to a final concentration of 100 µM. Mock-treated plants were soil-drenched without ABA. Aerial part of the plants used for the experiments was harvested and processed for BABA extraction 48 hpt Data represent the mean and standard

deviation (n = 3 biological replicates). All experiments were repeated in triplicate. Asterisks indicate significant differences as determined by a Student t -test: *P < 0.05; ***P < 0.001.

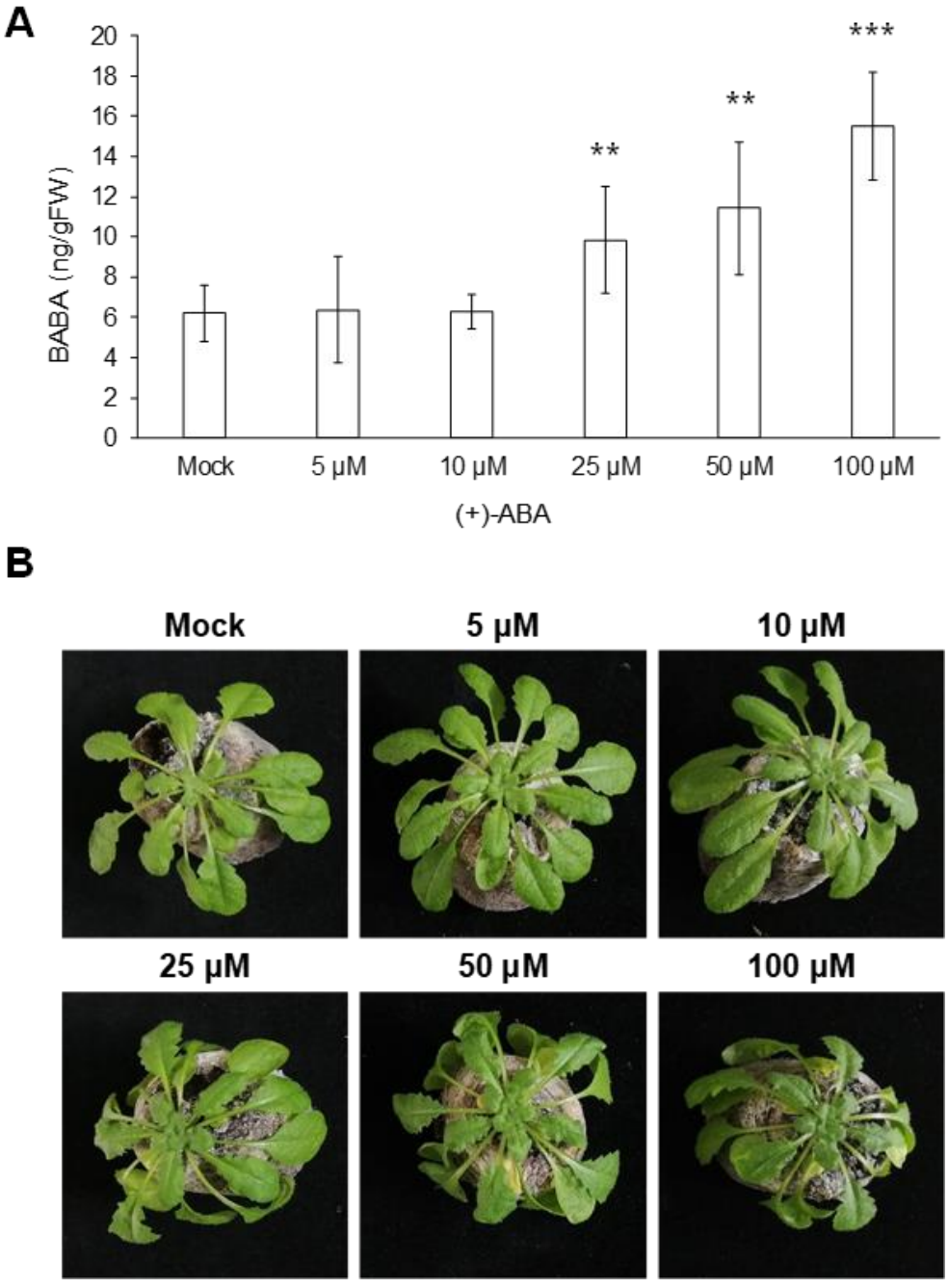

Fig. 4-3: (+)-ABA spray-treatment effect on BABA concentrations in Arabidopsis Col-0 plants. A: 5-week-old Arabidopsis Col-0 plants were sprayed with a progressive concentration series from 5 to 100 µM (+)-ABA solutions. Mock-treated plants were sprayed with the same solutions without (+)-ABA. Aerial part of the plant was harvested and processed for BABA extraction 48 and 72 hpt. Data represent the mean and standard deviation (n = 6 biological replicates). All experiments were repeated in triplicate. Asterisks indicate significant differences as determined by a Student t-test: **P < 0.01; ***P < 0.001. B: (+)-ABA spray-treatment effect on the phenotype of Arabidopsis Col-0 plants. (+)-ABA progressive concentration solutions induce senescence, evident by yellowish leaves and wilting of leaves, caused by stomatal closure.

4.3.3. BABA accumulation in *SnRK2s* mutants

To verify if ABA-dependent BABA accumulation is under the control of ABA-signaling, we soil-drenched plants with 100 µM (+)-ABA solution and assessed BABA concentrations 48 hpt in the ABA-insensitive *snrk2.2 snrk2.3* double mutant and *snrk2.6/ost1-3* mutant (Mustilli et al., 2002; Fujii et al., 2007; Fujii and Zhu, 2009; Zheng et al., 2010; Wang et al., 2018). Unfortunately, the strong dwarf phenotype of the plants did not allow to perform our experiments with the triple mutant *snrk2.2 snrk2. /snrk2.6* (Fujii and Zhu, 2009).

Interestingly *snrk2.2 snrk2.3* showed a (+)-ABA-dependent BABA induction 48 hpt (**Fig. 4-4A**). In the same way, *snrk2.6/ost1-3* mutant showed a wild-type accumulation of BABA after (+)-ABA treatment (**Fig. 4-4B**). *snrk2.2 snrk2.3* induced BABA to a lesser extent compared to wild type and *ost1-3*. Nevertheless, these data are not sufficient to correlate ABA-dependent BABA induction with ABA-signaling.

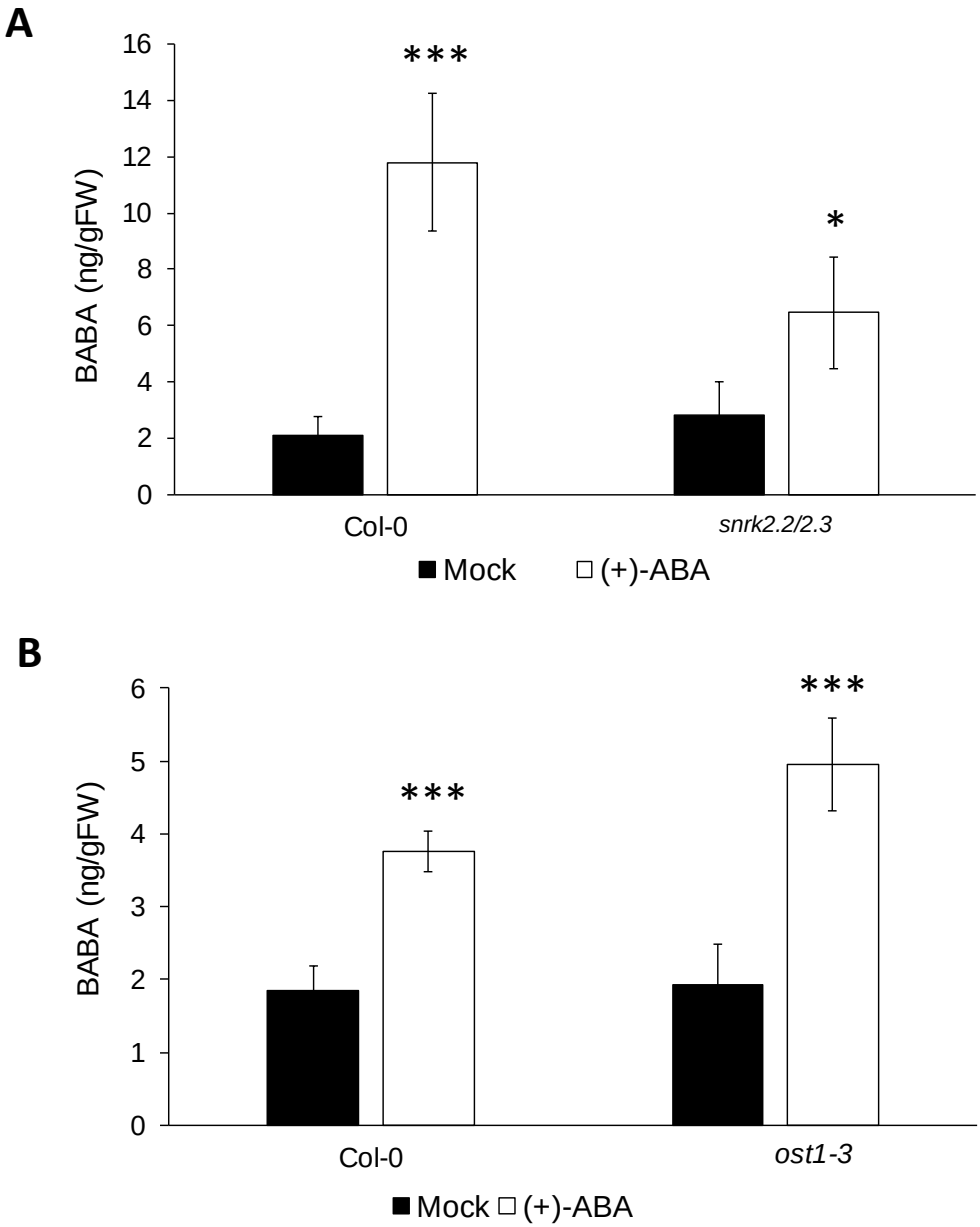

Fig. 4-4: (+)-ABA effects on BABA concentrations in *snrk2.2/snrk2.3* double mutant and in *ost1-3* mutant. A: 5-week-old Arabidopsis Col-0 and snrk2.2/snrk2.3 plants were soil-drenched with a (+)-ABA solution 100 µM final concentration. Mock-treated plants were soil-drenched with the solution without ABA. B: 5-week-old Arabidopsis Col-0 and ost1-3 plants were soil-drenched with a (+)-ABA solution 100 µM final concentration. Mock treated plants were drenched in soil with the solution without the corresponding ABA. Aerial part of the plant was harvested and processed for BABA extraction 48 and 72 hpt. Data represent the mean and standard deviation (n = 3 biological replicates). All experiments were repeated in triplicate. Asterisks indicate significant differences as determined by a Student t-test: *P < 0.05; ***P < 0.001.

4.4. Discussion

The activity of BABA as an inducer of resistance has been known for decades (Cohen et al., 2016). More recently, it became clear that plants are able to synthesize BABA themselves and BABA concentrations under different stresses, observed during both biotic and abiotic stresses (Thevenet et al., 2017). Plant produced BABA was even suggested to be a new plant hormone (Baccelli and Mauch-Mani, 2017). An important aspect of hormonal signaling is the interaction to coordinate responses. However, the lack of knowledge on signaling restrains to precisely define BABA as a plant hormone and the relation of BABA with other hormones.

Starting with this problem, we wanted to know the possible relations occurring between plant-made BABA and plant hormones. Therefore, we first treated plants with solutions of different plant hormones sprayed or soil-drenched. None of the plant hormones was able to increase BABA concentrations. Despite the importance of SA-signaling in BABA-IR, SA did not increase BABA levels. The same fact was observed upon MeJA and ACC treatment. A possible explanation for this pattern stems from the data on applied BABA, which seems to act as a trigger for SA, JA and ET signaling (Baccelli and Mauch-Mani, 2016). In fact, BABA activates SA signaling to induce resistance against bacteria and other pathogens, via induction of PR proteins synthesis and phenocopy HR at the site of pathogen growth (Zimmerli et al., 2000). BABA primes *PR-1* expression only during the post-challenge primed phase (Zimmerli et al., 2000). By contrast, our experiments were performed only in unstressed conditions, leaving open the possibility that defense-related hormones JA, ET and SA may boost BABA concentrations after challenge application. Furthermore, BABA primes the expression of JA-activated genes, such as *LOX-9* and *PR-4*, in grapevine against *P. viticola*, while boosts *ETR1* transcription in drought stressed potatoes (Hamiduzzaman et al., 2005; Sós-Hegedűs et al., 2014). Furthermore, BABA application on Arabidopsis led to a transient induction of IAA at 24 hpt (Pastor et al., 2014).

Interestingly, the only hormone inducing BABA was ABA, although this induction was found only in soil-drench treatment. The lack of induction found in the ABA spray treatment may be dependent on the half concentrations present in the racemic powder. The biologically active enantiomer (+)-ABA induces BABA also in spray treatment. (+)-ABA strongly induce senescence and leaf wilting in 5-week-old Arabidopsis Col-0 in both spray (**Fig. 4-3B**) and soil drench (**Fig. S4-6**). Furthermore, the higher the concentrations applied, the higher the concentrations of BABA detected. This phenotype may be due not directly to the (+)-ABA

application, but instead to the physiological changes induced, such as senescence. However, ABA application can induce modifications in amino acid content (Andres and Smith, 1976; Nambara et al., 1998; Kovács et al., 2011). Hundred μM ABA application has been shown to increase proline concentrations in Arabidopsis WT during abiotic stress, while the biosynthetic mutant *aba2-2* accumulates significantly less proline compared to wild type (Nambara et al., 1998). Furthermore, ABA up-regulates asparagine synthase and increases asparagine concentrations in wheat (Wang et al., 2005; Kovács et al., 2011). ABA also increases secondary metabolites, such as in maize the DIMBOA and the ester of caffeic acid and quinic acid, chlorogenic acid, involved in defense against insects (Erb et al., 2009). The data presented here suggest that BABA is a non-proteinogenic amino acid and a defense-related molecule induced by ABA.

To confirm the ABA-dependent BABA induction is under the control of the ABA signaling, we analysed at BABA concentrations in *SnRK2s* mutants. SnRK2s are a key turning point in the response to ABA, integrating the signal into action (**Fig. 4-1**) (Hirayama and Umezawa, 2010). The complexity of the BABA receptors PYL/PYR/RCARs, whose family is composed of fourteen members (Ma et al., 2009), the wide number of PP2CA proteins (Xue et al., 2008) and of AREB/ABF transcription factors (Singh and Laxmi, 2015) led us to prefer the three SnRK2s involved in ABA signaling. The *snrk2.6/ost1-3* showed a wild-tape (+)-ABA-dependent BABA induction, while the *snrk2.2 snrk2.3* double mutant induced BABA to a lesser extent. This low induction could depend on compensation effect by SnRK2.6, although not strong enough to restore BABA concentrations to wild type levels. The BABA concentrations found in *snrk2.6/ost1-3*, in both control and (+)-ABA treatments, are lower compared to the data reported in literature and in this work. This may be due by a loss in sensibility in the TQ-S HPLC.

SnRK2s play different roles in ABA signaling. Loss of function mutants of SnRK2.6/Ost1 failed in expressing ABA-dependent stomatal closure (Mustilli et al., 2002). The *snrk2.2* and *snrk2.3* single mutants, by contrast, did not express a clear ABA-insensitive phenotype and this has been attributed to a compensation effect. In fact, the double mutant *snrk2.2 snrk2.3* showed altered seed dormancy, germination, and growth (Fujii et al., 2007). Nevertheless, although both snrk2.6 and snrk2.2 snrk2.3 double mutant showed a lower expression of ABA-dependent genes, the induction was not totally blocked and development was similar to wild type (Yoshida et al., 2002; Fujii et al., 2007). Consequently, to confirm that BABA induction is under the control of the ABA-signaling, the use of the *SnRK2s* triple mutant would be necessary. In fact,

the triple mutant *snrk2.2 snrk2.3 snrk2.6* expresses a low seeds production and dwarf phenotype, faster water loss and totally blocked ABA-dependent genes induction (Fujii and Zhu, 2009). The triple mutant was also insensitive in ABA-dependent blocking of seed germination and root growth (Gosti et al., 1999). However, we cannot use the triple mutant for our experiments, as they require a certain quantity of fresh material impossible to obtain with this mutant. Therefore, further experiments are needed to confirm that BABA induction after ABA-treatment is dependent on ABA-signaling.

4.5. References

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4.6. Supplementary material

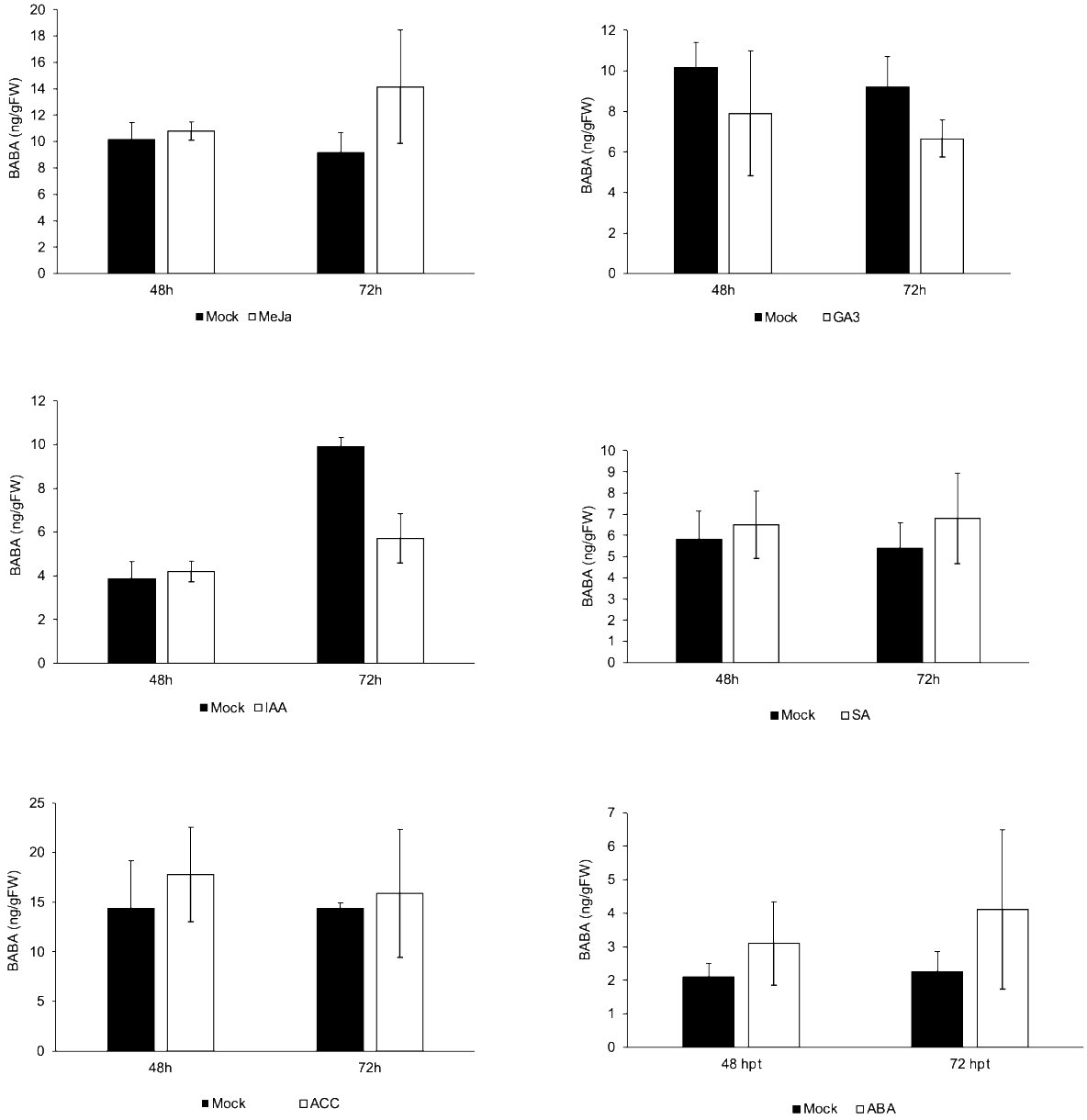

Fig. S4-1: Hormonal spray-treatment effect on BABA concentrations in Arabidopsis Col-0 plants. 5-week-old Arabidopsis Col-0 plants were sprayed with a series of 100 µM phytohormone solutions. Mock treated plants were sprayed with the solutions without the corresponding hormone. Aerial part of the plant was harvested and processed for BABA extraction 48 and 72 hpt. Data represent the mean and standard deviation (n = 3 biological replicates). Differences between control and treatment have been determined by a Student t -test.

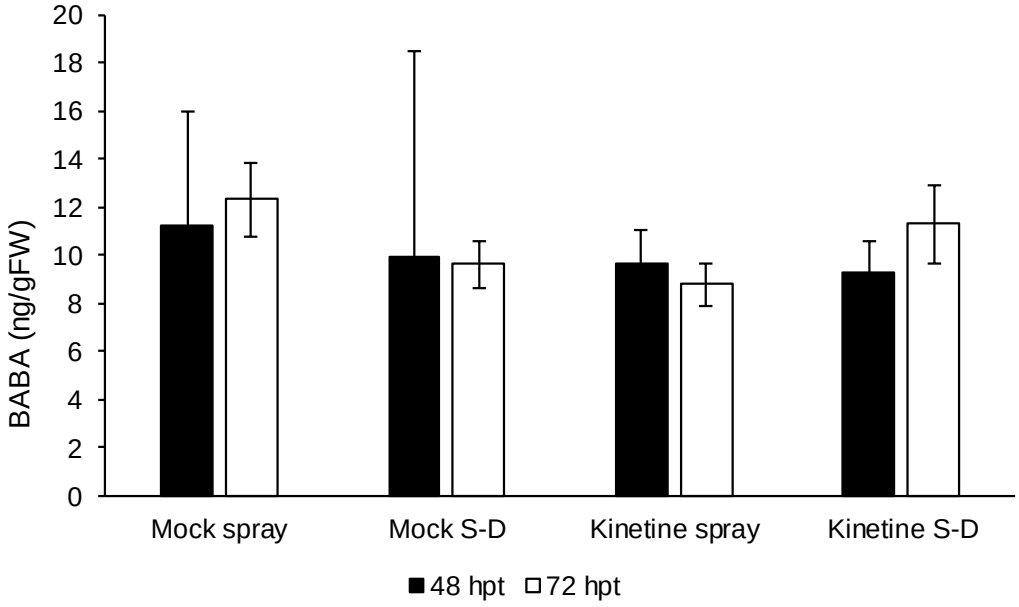

Fig. S4-2*:* Kinetine spray- and soil-drench-treatment (S-D) effect on BABA concentrations in Arabidopsis Col-0 plants. 5-week-old Arabidopsis Col-0 plants were sprayed with a series of 100 µM kinetine solutions. Mock treated plants were sprayed with the solutions without kinetine. Aerial part of the plant was harvested and processed for BABA extraction 48 and 72 hpt. Data represent the mean and standard deviation (n = 3 biological replicates). All experiments were repeated in triplicate. Differences between control and treatment have been determined by a Student t -test.

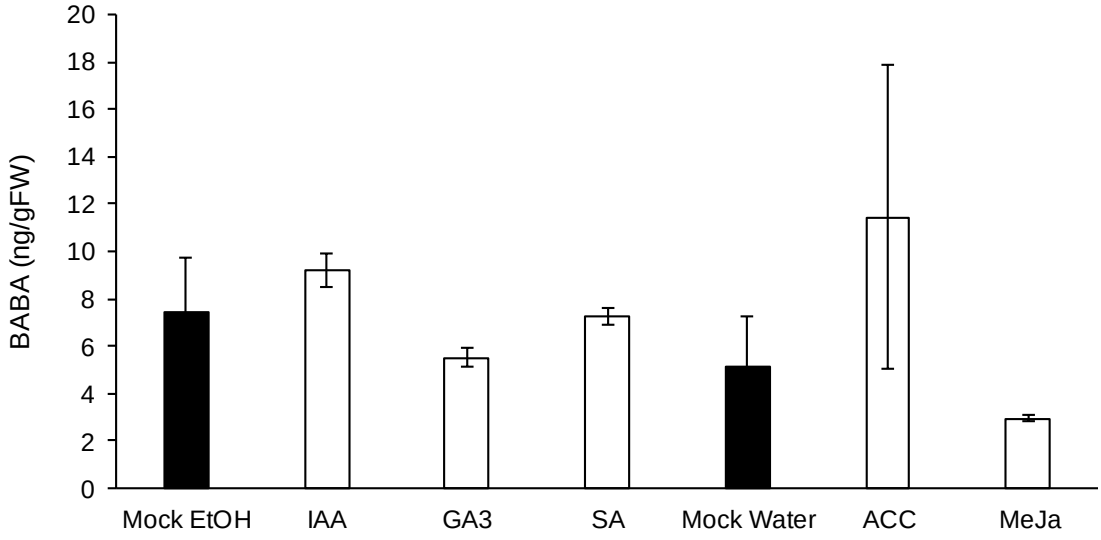

Fig. S4-3: Hormones soil-drench-treatment effect on BABA concentrations in Arabidopsis Col-0 plants 72 hpt. 5-week-old Arabidopsis Col-0 plants were drenched in soil with a series of 100 µM phytohomrone solutions. Each hormonal treatment is placed by the side of the corresponding mock solution. Mock EtOH represents control solution with an ethanol concentration equal to that used for dissolving powders of hormones. Mock Water represents solutions made exclusively with mQ water. Aerial part of the plant was harvested and processed for BABA extraction 48 and 72 hpt. Data represent the mean and standard deviation (n = 3 biological replicates). All experiments were repeated in triplicate. Differences between control and treatment have been determined by a Student t -test.

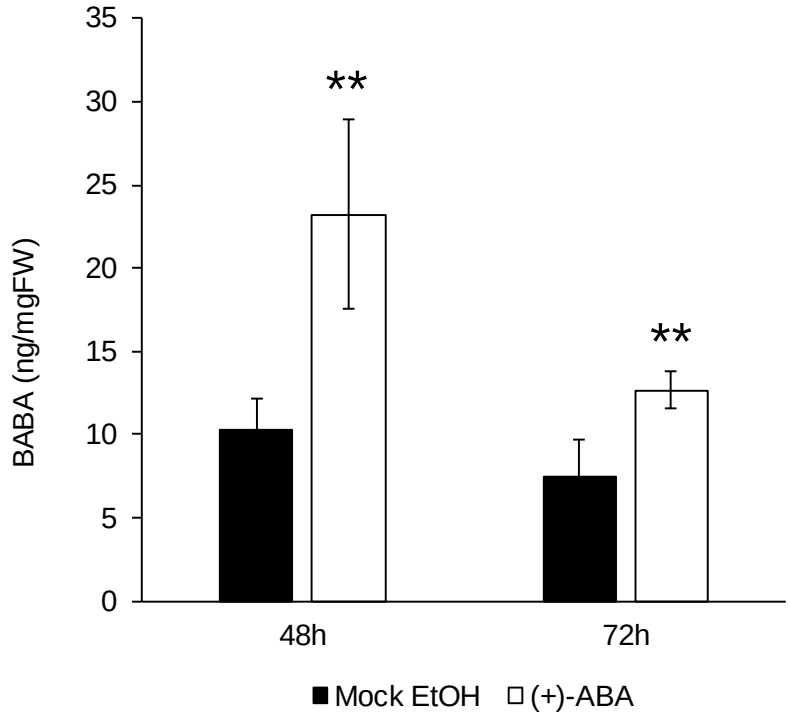

Fig. S4-4: (+)-ABA soil-drench-treatment effect on BABA concentrations in Arabidopsis Col-0 plants 48 and 72 hpt. 5-week-old Arabidopsis Col-0 plants were drenched in soil with a series of 100 µM (+)-ABA solution and harvested and processed for BABA extraction 48 and 72 hpt. Data represent the mean and standard deviation (n = 6 biological replicates). All experiments were repeated in triplicate. Asterisks indicate significant differences as determined by a Student t -test: **P < 0.01.

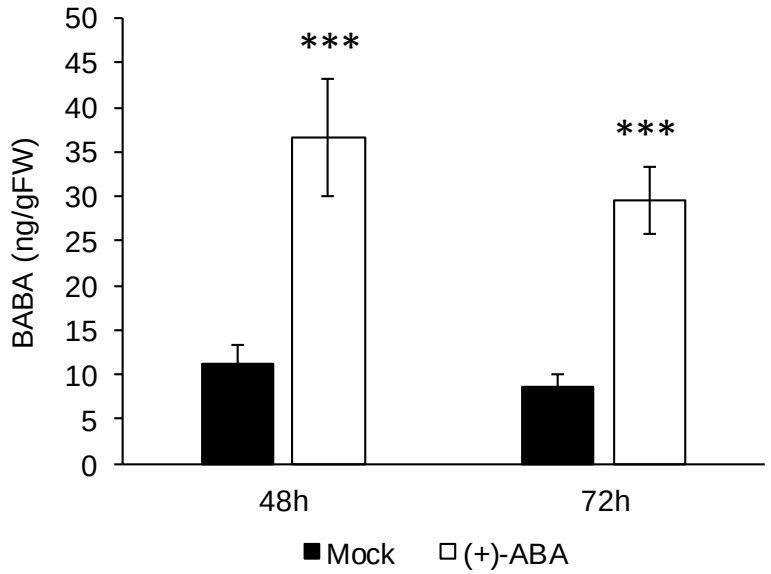

Fig. S4-5: (+)-ABA spray-treatment effect on BABA concentrations in Arabidopsis Col-0 plants 48 and 72 hpt. 5-week-old Arabidopsis Col-0 plants were sprayed with a 100 µM (+)-ABA solution and harvested and processed for BABA extraction 48 and 72 hpt. Data represent the mean and standard deviation (n = 6 biological replicates). All experiments were repeated in triplicate. Asterisks indicate significant differences as determined by a Student t -test: ***P < 0.001.

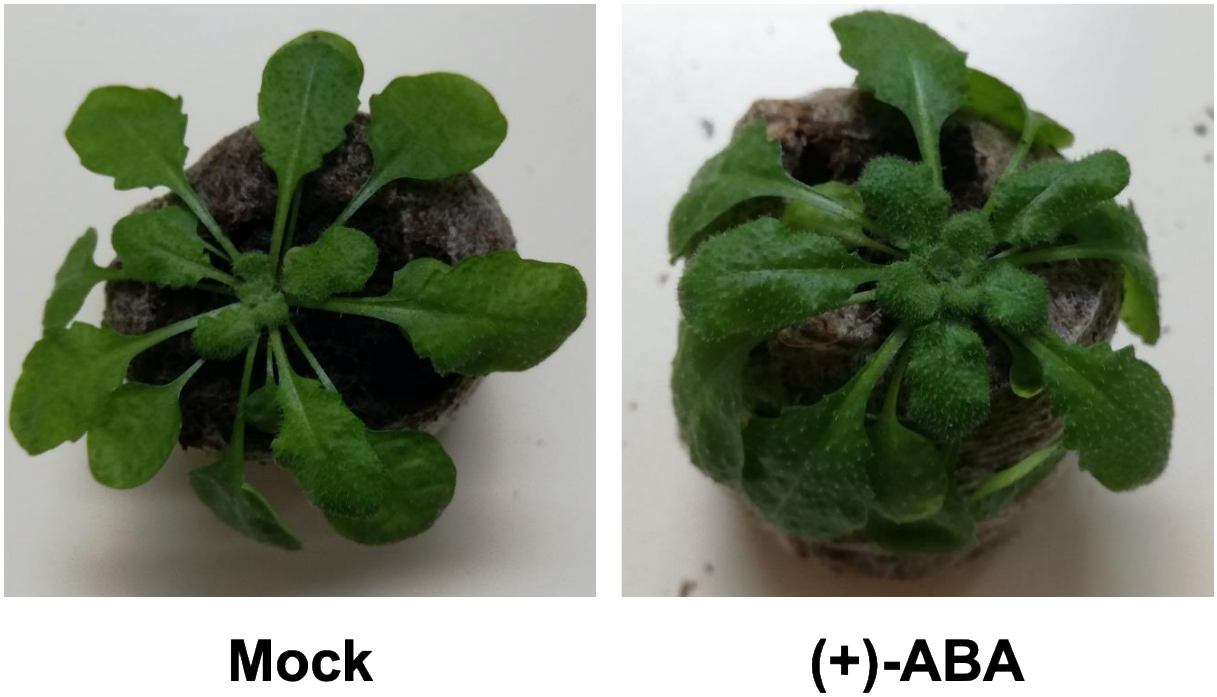

Fig. S4-6: Effects of drenching into soil a 100 µM final concentration (+)-ABA solution on Arabidopsis Col-0.

5. Studying BABA subcellular localization using copper-catalyzed azide–alkyne cycloaddition for the synthesis of fluorescent-labeled BABA molecules to visualize with confocal microscopy

Francesco Stefanelli, Damien Thevenet, Guillaume Gouzerhand Brigitte Mauch-Mani

The movement of β-aminobutyric acid (BABA) in plants has been documented in several works. When applied to plants, BABA moves both acropetally and basipetally, preferably towards younger and infected tissues. In these tissues, BABA activates successively defense response. However, it is not clear the subcellular localization of BABA. To solve this problem, we decided to use fluorescence microscopy. For this purpose, we produced tagged-BABA molecules (TAG_{n1-6}), adding an alkyne group at each BABA chemical group. We tested the induced-resistance capacity of TAGs, and we selected TAG4 for further microscopic experiments. We incubated Arabidopsis seedling with a TAG4 solution for two days. Successively, we coupled TAG4 with the fluorophore Alexa fluor 488 ® in a copper-catalyzed azide-alkyne cycloaddition (CuAAC), a technique used to study glycobiology of the cell wall. Results showed that BABA localized mainly in the cell wall and globular subcellular structures, possibly involved in vesicle trafficking. These results were similar to TAG5 and proline-TAG5 as well. These data, although pioneering, are not sufficient to conclude the exact subcellular localization of BABA.

5.1. Introduction

BABA is one of the most studied priming agents, effective against biotic and abiotic stresses, and recently also discovered to be synthesized by plants (Cohen et al., 2016; Thevenet et al., 2017). In the first publications concerning BABA, it was observed both the movement and the subcellular localization of BABA (Cohen, 1994; Cohen et al., 1994; Jakab et al., 2001). The foliar spray of a BABA solution increased *PRs* transcripts in both tobacco and tomato leaves. However, the injection of BABA into stems of tobacco plants generated the opposite effect, although plants appeared well protected (Cohen, 1994; Cohen et al., 1994). Furthermore, BABA-IR was more prominent in upper than in lower leaves, in both tomato and tobacco (Cohen, 1994; Cohen et al., 1994). In addition, BABA treatment three days before *P. infestans* inoculum on all leaves of tomato plants conferred a significant high level of protection in every single leaf (Cohen and Gisi, 1994). By contrast, treating with BABA only the first three lower leaves, no significant protection was detectable in the leaf above the treated ones (Cohen and Gisi, 1994). Similarly, soil-drenched BABA solution conferred a high level of protection to tomato against late blight, although axial proximal leaflets were more protected than axial distal leaflets (Cohen and Gisi, 1994). This irregular pattern of protection might be associated with the translocation of BABA from one tissue to another (Cohen and Gisi, 1994). To confirm this hypothesis, Cohen and Gisi (1994) applied labeled ^{14}C-BABA solution droplets on old leaves of tomato plants and they looked at radioactivity one day after treatment. Interestingly, autoradiography images showed that apart from treated leaves, ^{14}C-BABA was detectable only in newly developed leaves and not in older leaves (**Fig. 5-1.A**) (Cohen and Gisi, 1994). Furthermore, the concentrations of the label decreased over time in treated leaves and increased in untreated leaves, suggesting an acropetally movement of sprayed BABA (Cohen and Gisi, 1994). At the same time, soil-drench applied ^{14}C-BABA was detected in all plant tissues one day after treatment. These data showed that soil-drench applied BABA also moved in plants basipetally (Cohen and Gisi, 1994). By side, the basipetally and acropetally translocation of BABA was also confirmed in lettuce, where the translocation of ^{14}C-BABA was associated with the induced resistance against *B. lettucae* (Cohen et al., 2010). Jakab et al. (2001) also demonstrated BABA movement in Arabidopsis accession Ws-0 using soil-drench ^{14}C-BABA and the preference for younger tissues as well (**Fig. 5-1.B**). Finally, isolating protoplasts from *Arabidopsis thaliana* ecotype Ws-0 plants treated with ^{14}C-BABA, Jakab et al. (2001)

demonstrated that BABA accumulates in the organellar/membrane fraction and not within the cell wall (**Fig. 5-1.C**). Nevertheless, these data were in contrast with Cohen and Gisi (1994) and Cohen et al. (1999), which found that sprayed ^{14}C-BABA can be isolated from the soluble fraction but it can also be retained by the cell wall.

Information on the localization of plant-produced BABA come from Balmer et al. (2019). In this work, authors looked at the concentration of BABA in locally infected leaves with *Pst AvrRpt2* and P. *cucumerina* and systemic non infected leaves. Locally infected leaves showed high concentrations of BABA compared to unchallenged plants (Balmer et al., 2019). Nevertheless, neither *Pst AvrRpt2* nor *P. cucumerina* infected plants show BABA induction in systemic leaves. Furthermore, no increased BABA concentrations after infection with *P. cucumerina* were detected in roots 48 or 72 hpt (Balmer et al., 2019). Unfortunately, no data are currently known for the subcellular localization of BABA.

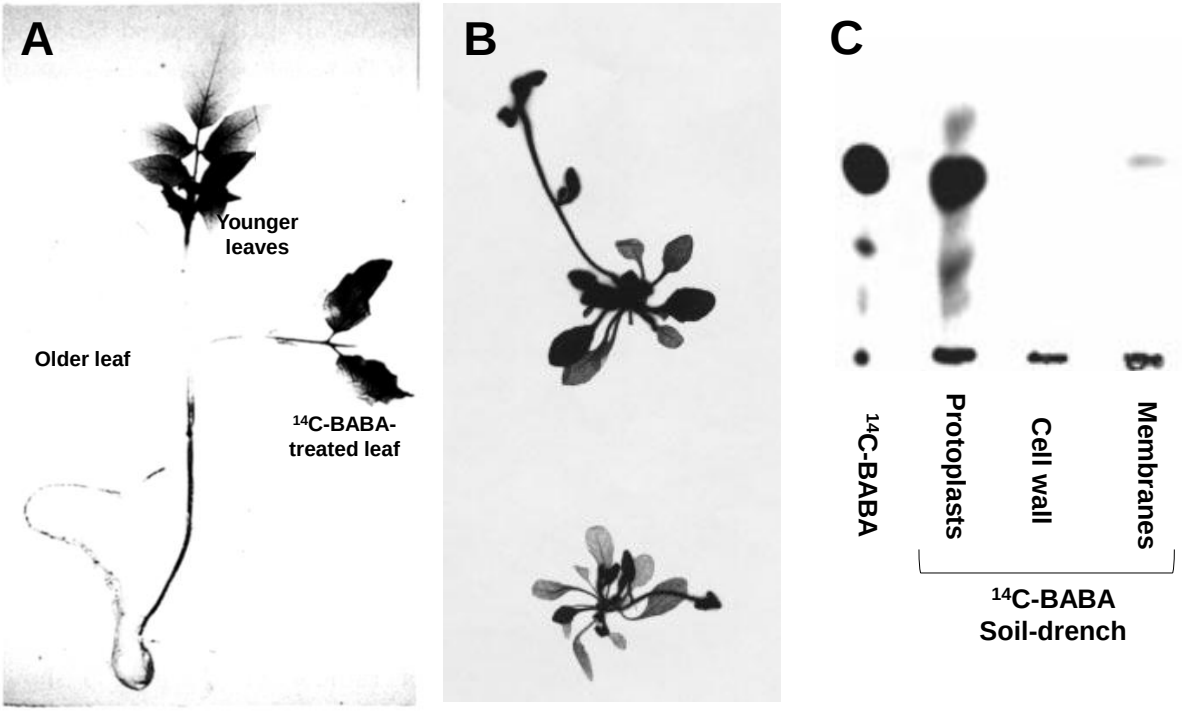

Fig. 5-1: BABA movement in plants. A: autoradiograph of sprayed ^{14}C-BABA-treated tomatoes 24 hours post treatment. Image from Cohen and Gisi (1994). **B**: autoradiogram of soil-drench ^{14}C-BABA-treated Arabidopsis Ws 48 hpt. Image from Jakab et al. 2001. **C**: autoradiogram of layer chromatogram of ^{14}C-labeled BABA. Lane 1: ^{14}C-labeled BABA; lane 2: protoplast of soil drench ^{14}C-treated BABA Arabidopsis Ws. Lane 3: cell wall fraction of soil drench ^{14}C-treated BABA Arabidopsis Ws. Lane 4: membrane and organelle fraction of soil drench ^{14}C-treated BABA Arabidopsis Ws. Image from Jakab et al. 2001

In this work we wanted to employ an alternative technique to get new insights in BABA subcellular localization. We decided to use copper-catalyzed azide–alkyne cycloaddition (CuAAC), also known as click.chemistry (Rostovtsev et al., 2002; Meldal and Tornøe, 2008) for producing fluorescent BABA molecules to visualize in confocal microscope *in vivo*. We used alkyne-modified BABA analogs (TAGs) produced in the laboratory of organic chemistry of the University of Neuchatel by Damien Thevenet (**Fig. 5-2**). Thevenet added an alkyne group at each functional group of the BABA molecule, naming TAG1 and TAG6 the BABA molecules modified in the methyl group, TAG2 and TAG3 in the carboxylic group and TAG4 and TAG5 the BABA molecules tagged in the ammine group (**Fig. 5-2**). After different chemical analysis (data not shown), TAG1, TAG4, TAG5 and TAG6 were furnished for further experiments for their stability and the degree of purity. In fact, TAG2- and TAG3-treated Arabidopsis showed elevated levels of endogenous BABA due to the cleavage of the alkyne function from the carboxyl and amide groups (data not shown). Successively, for the set-up of CuAAC, we decided to use the fluorochrome Alexa Fluor 488® picolyl azide (Alexa 488), which binds TAGs in a reaction catalyzed by Cu(I), forming a BABA-Alexa 488 complexes that can be visualized *in vivo* in confocal microscopy (**Fig. 5-2**). Confocal microscopy experiments showed that TAG4 localizes in root cell walls but not in leaves. Furthermore, we found the presence of TAG4 in intracellular globular structures. However, this pattern was also seen applying the TAG5 and an alkyne-modified Proline-TAG5. Nevertheless, our results are not sufficient to definitely defined BABA subcellular localization and movement in plants but suggest a possible incorporation in root cell wall and vesicle trafficking.

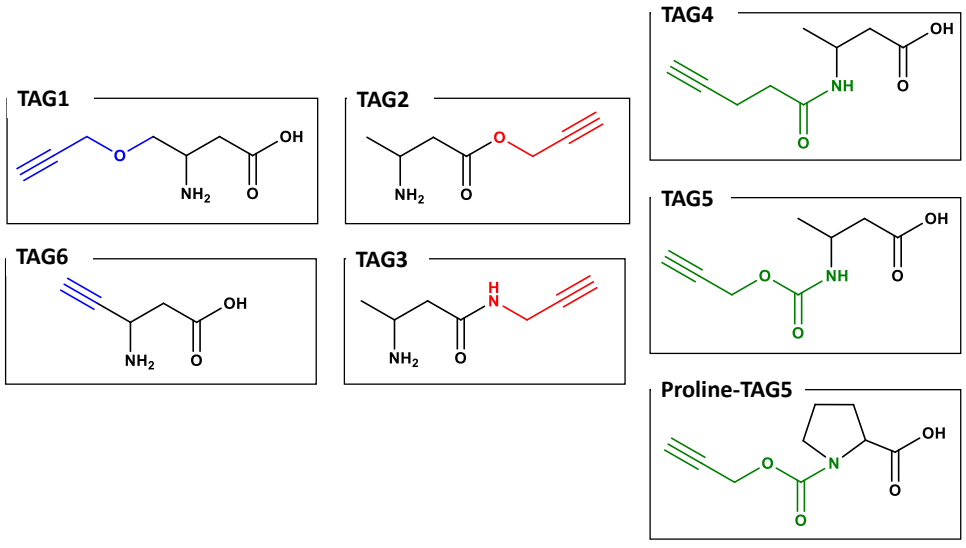

Fig. 5-2: Structures of the alkine-modified BABA molecules and of Proline-TAG5.

5.2. Materials and methods

5.2.1. Plant Material and Growth Conditions

Seeds of Arabidopsis Col-0 plants used for resistance assays were multiplied from a stock purchased at Lehle Seeds, Round Rock, TX, USA and sown on Jiffy-7 peat pellets and stratified at 4 °C in darkness for 2 days before being placed in controlled growth cabinets. Seedlings were cultivated under standard growth conditions: 21 °C day 18 °C night with 8h-day (light intensity of 100 μmol/m^2/s) and 16h-night (short-day-light conditions), at ~70 % relative humidity.

For plants used for microscope experiments, Arabidopsis Col-0 seeds were sowed on half-strength Murashige and Skoog Medium (MS; Sigma-Aldrich) with 0.7% of phytoagar (Duchefa Biochimie) and stratified at 4 °C in darkness for 2 days before being placed to controlled growth cabinets at same conditions as mentioned before.

5.2.2. Pathogen cultivation and resistance assays

2- to 3-week-old plants were sprayed with a suspension of 5 x 10^5 conidiospore mL^{-1} of *Hpa* strain NOCO. Fifteen randomly selected plants were put in 5 mL of tap water, shaken to detach spores from the conidiophores, and the suspension obtained transferred in a new tube to count for each treatment the number of conidiospores per mL (Glazebrook and Weigel, 2002). Differences were analyzed with Student's t-test. Also, we evaluated BABA and TAG-induced resistance with fifteen randomly selected plants were also collected at 6 d after spray inoculation and stained with lactophenol–trypan blue and scored using light microscopy (Nikon E800 Microscope). Colonization levels were assigned to four classes: I, no pathogen growth; II, hyphal colonization without conidiophores; III, hyphal colonization with conidiophores and sporadic oospores; and IV, extensive colonization, conidiophores, and frequent oospores. Differences between classes were assessed using Fisher's exact test with Microsoft Excel.

Pst DC3000 was grown overnight at 28 ˚C in King's medium B with Rifampicin (50 mg/mL) to OD600= 0.6–0.9. Bacteria were harvested by centrifugation (3000 *g*) and diluted in 10 mM $MgSO_4$. Infection with *Pst* was performed by dipping aerial part of 5-week-old Arabidopsis Col-0 in a suspension of 10^7 colony-forming units (CFU) mL^{-1} (OD600 = 0.02) in 10mM $MgSO_4$ 0.02% Silwet-L77. The ground aerial part of the plant was re-suspended in an adequate volume of 10 mM $MgSO_4$ and the suspension cultivated in King's agar B containing 50 μg mL^{-1} of Rifampicin at 28 ˚C until colony formation. The bacterial growth was monitored

at 0, 2 and 4 days after infection and expressed as cfu $mgFW^{-1}$. The results were printed out as growth curves. Statistical analysis was performed using a Tukey HSD (Honestly Significant Difference) test (α=0.05).

Five-week-old *A. thaliana* Col-0 plants were drop inoculated with 10 µl of a spore suspension (5×10^6 spores mL^{-1}) of *P. cucumerina* BMM in half strength (12 g L^{-1}) Potato Dextrose Broth (PDB, Sigma-Aldrich), and incubated in 100% relative humidity conditions. Disease symptoms were evaluated 6 days after inoculation by determining the average lesion diameter. Differences between treatments were analyzed with a Tukey HSD (Honestly Significant Difference) test (α=0.05). The pathogen was cultivated on half-strength PDB agar 0,7% in dark at constant temperature of 24 ˚C.

5.2.3. Chemical treatments and microscopic visualization

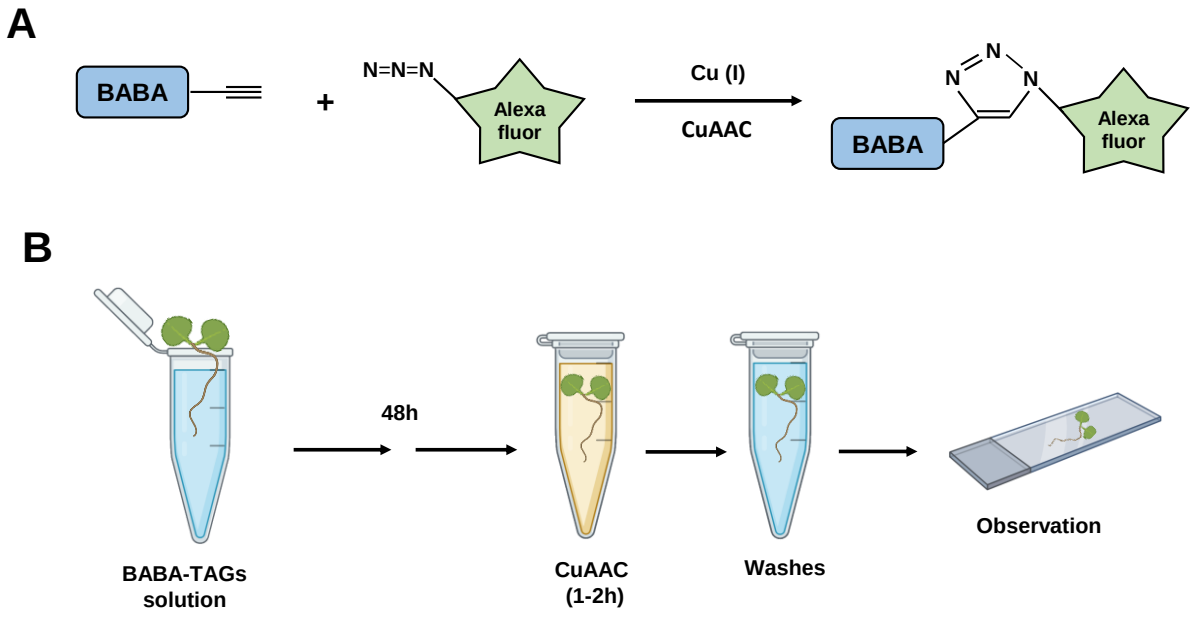

Fig. 5-3: Methodology for the set-up of copper-catalyzed azide–alkyne cycloaddition (CuAAC) with BABA-TAGs and Alexa fluor 488 in Arabidopsis. A: The bind of the alkine modified BABA (TAGs) with Alexa fluor 488 containing an azide group produce a complex able to emit fluorescence in a CuAAC reaction catalyzed by Cu (I). **B**: Arabidopsis seedlings were incubated in a TAG solution for 48 hours and then put in the reaction buffer for the set-up of CuAAC. After washes, plants were ready for confocal microscope observations.

TAGs and Proline-TAG5 (**Fig. 5-3**) were synthetized by Damien Thévenet in the Laboratory of Organic Chemistry of the University of Neuchâtel. For resistance tests, Arabidopsis Col-0 plants were soil-drenched with a solution of TAGs and BABA for a final concentration of 150 µM. Control plants were treated with an equal volume of water.

For CuAAC experiments and further microscope visualization, 2 weeks-old Col-0 seedlings grown on half MS 0.7% agar were transferred in a solution of 150 µM of TAGs or 1.5 mM of TAG4 and in tap water as negative control, for two days until microscopic observation in order to incorporate TAGs (**Fig. 5-3**). TAGs and control-treated seedlings were rinsed twice in milliQ water and after put in a staining solution with 5 µM Alexa Fluor 488® Picolyl azide dye (Thermo Fisher scientific, Waltham, Massachusetts, United States) for 1 h in the dark to perform the click-catalyzed click reaction (**Fig. 5-3**). Samples were rinsed again several times in water before imaging, washing out unspecific labelling (**Fig. 5-3**). Images were collected with a TCS SP5 II confocal laser scanning microscope (Leica Biosyntems, Wetzlar, Germany) based in the Swiss Center for Electronics and Microtechnology (CSEM, Neuchâtel, Switzerland). A whole Arabidopsis Col-0 seedling has been transferred onto microscope slide and covered with coverslip. Digital images were acquired using LAS AF (version: 2.6.3 build 8173). TAGs-Alexa 488 complexes were visualized using 488 nm excitation and an emission at 525 nm.

5.3. Results

5.3.1. Selecting alkyne-tagged-BABA analogues for their induced resistance capacity

Before testing the different TAGs for *in vivo* application and microscope visualization, we decided to find the closest analog to BABA in inducing resistance. Therefore, the TAGs provided were screened looking at their resistance-inducing capacity against the oomycete *Hpa* NOCO, measuring thus pathogen sporulation and comparing them with BABA. Results indicate that all TAGs were able to induce resistance against *Hpa*, but TAG4 showed the lowest sporulation activity (**Fig. 5-4**). For this reason, TAG4 was chosen for next resistance tests, but the induced-resistance activity against *Hpa* was measured again with infection-rating method, confirming the resistance inducing capacity, although less effective compared to BABA (**Fig. S5-1**).

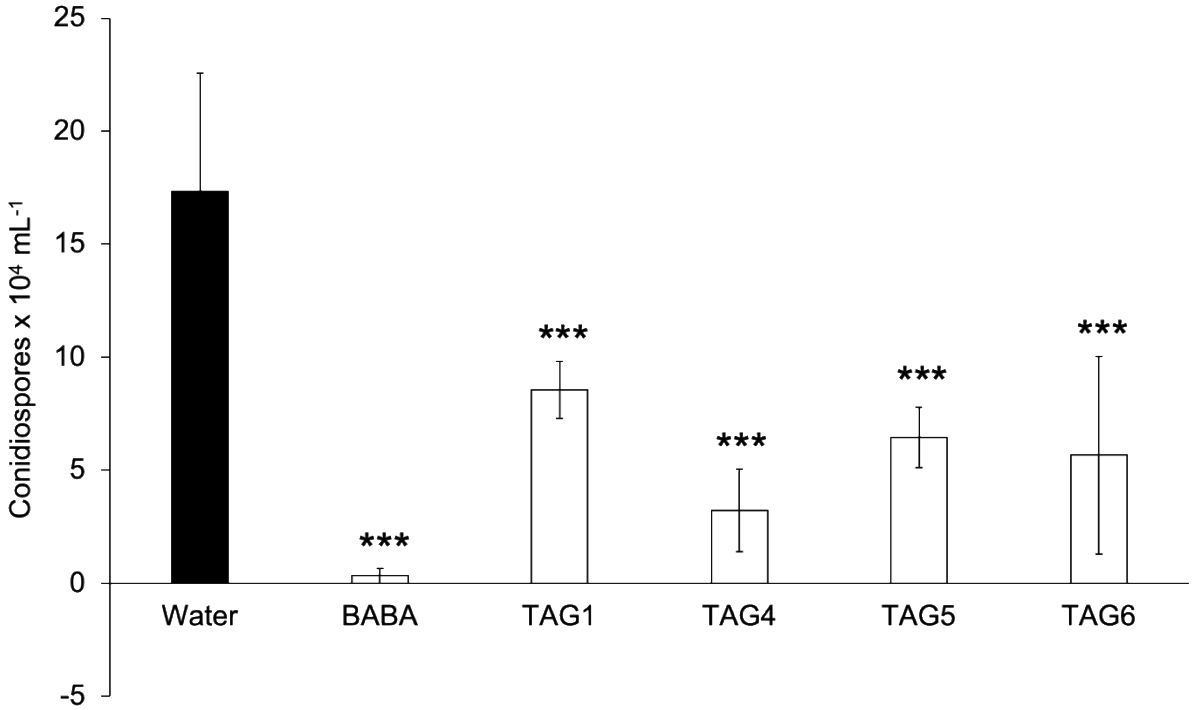

Fig. 5-4: Tagged-BABA analogues resistance inducing capacity against *Hyaloperonospora arabidopsidis* NOCO. Fifteen two-weeks-old BABA and TAGs-treated Arabidopsis Col-0 seedlings have been inoculated with *Hpa* Noco. Seven days after infection, plants have been put in 5 mL of tap water and shacked to detach conidiospores from conidiophores. Conidiospores of *Hpa* Noco have been counted with a hemocytometer. TAG4 show the best performances among the different TAGs. Asterisks indicate significant differences as determined by a Student's t-test: ***P < 0.001.

The inducing-resistance capacity of TAG4 was further investigated using the hemibiotrophic pathogen *Pst* DC3000 and the necrotrophic pathogen *P. cucumerina*. Plants treated with 150 µM TAG4 showed a reduced bacterial load at both 2- and 4-days post infection, similarly as BABA-treated plants (**Fig. 5-5.A**). On the other side, TAG4 reduced the lesion diameter in *P. cucumerina*-infected leaves, although slightly more than in BABA-treated plants (**Fig. 5-5.B**). Taken together, these results confirmed the resistance inducing-capacity of TAG4 in Arabidopsis.

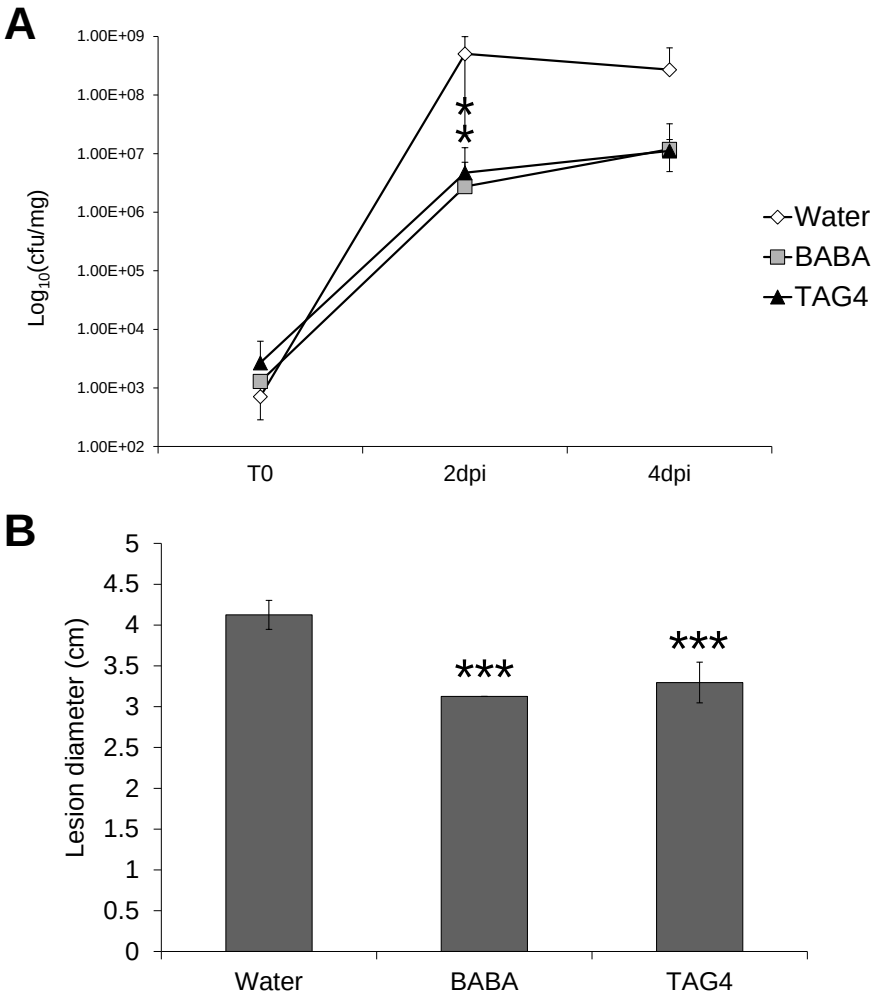

Fig. 5-5. Induced-resistance capacity of BABA-TAG4 against necrotrophic and hemibiotrophic pathogens. A: induced resistance of BABA and TAG4 against *Pst* DC3000 compared to water treated plants. **B**: Lesion diameter (expressed in cm) of BABA treated and BABA-TAG4 treated plants. Asterisks indicate significant differences as determined by Tukey HSD test (α=0.05).: *P < 0.05; ***P < 0.001.

5.3.2. BABA-TAG4 shows a root cell wall localization pattern

After assessing the resistance-inducing capacity, TAG4 was used to better understand localization of BABA in plants. A CuAAC with Alexa 488 was performed two days after incubating Arabidopsis Col-0 solution with a 150 μM of TAG4 solution. Confocal images showed a distinct localization pattern for TAG4: in epidermal root cells and root hairs (**Fig. 5-6.A**). In general, BABA-TAG4 localized exclusively at cell walls, except for globular structures of cytoplasmic compartmentation not found in control (**Fig. 5-6.A**). Interestingly, differently from roots, no labelling was detected in leaves (**Fig. 5-6.B**).

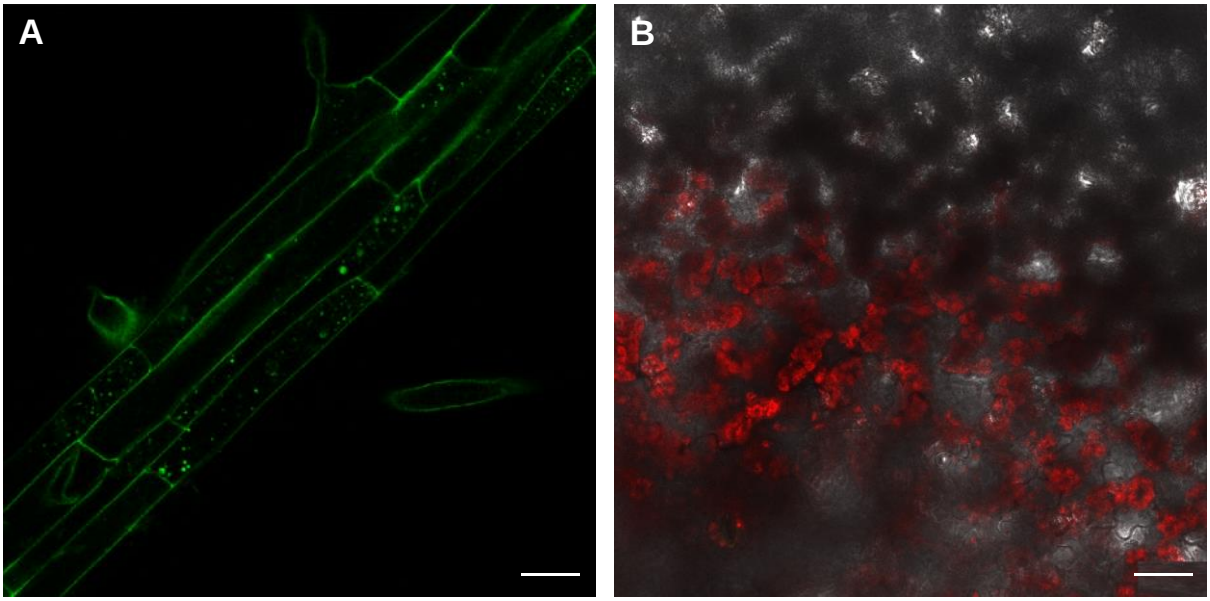

Fig. 5-6: Click-labeling and fluorescence imaging of Arabidopsis seedlings treated with a 150 µM TAG4 solution. A: fluorescent picture represents the roots and root hairs of Arabidopsis seedlings incubated in a TAG4 solution 48 hours post-treatment with labelling in cell wall of epidermidis and root hairs. Globular structures also have been detected. **B**: merged picture shows leaves of Arabidopsis seedlings incubated 48 hours with TAG4, where no labeling have been detected. Scale bar correspond to 25 µm.

Additionally, we treated plants with a solution ten-fold higher concentrated TAG4 (1.5 mM). In mock-treated plants, no fluorescence was detected (**Fig. 5-7.A**). In TAG4-treated plants, fluorescence was visualized in cell wall of root hairs and root epidermal cells (**.B and C**). The presence of undefined spots was also visualized at this higher concentration treatment (**Fig. 5-7.B and C**). At this concentration, a more intracellular localization has been seen in root hair (**Fig. 5-7.A**). Furthermore, labelling of hypocotyl cell wall has also been shown, but differently from lower root zones does not show intracellular globular accumulations (**Fig. 5-7**Errore. L'origine riferimento non è stata trovata.**.D**). Some root cells show a more diffuse staining pattern, although difficult using only CuAAC to determine its nature (**Fig. 5-7.E**) Nevertheless, no localization in leaves have been detected after 1.5 mM TAG4 treatment (**Fig. 5-7.F**).

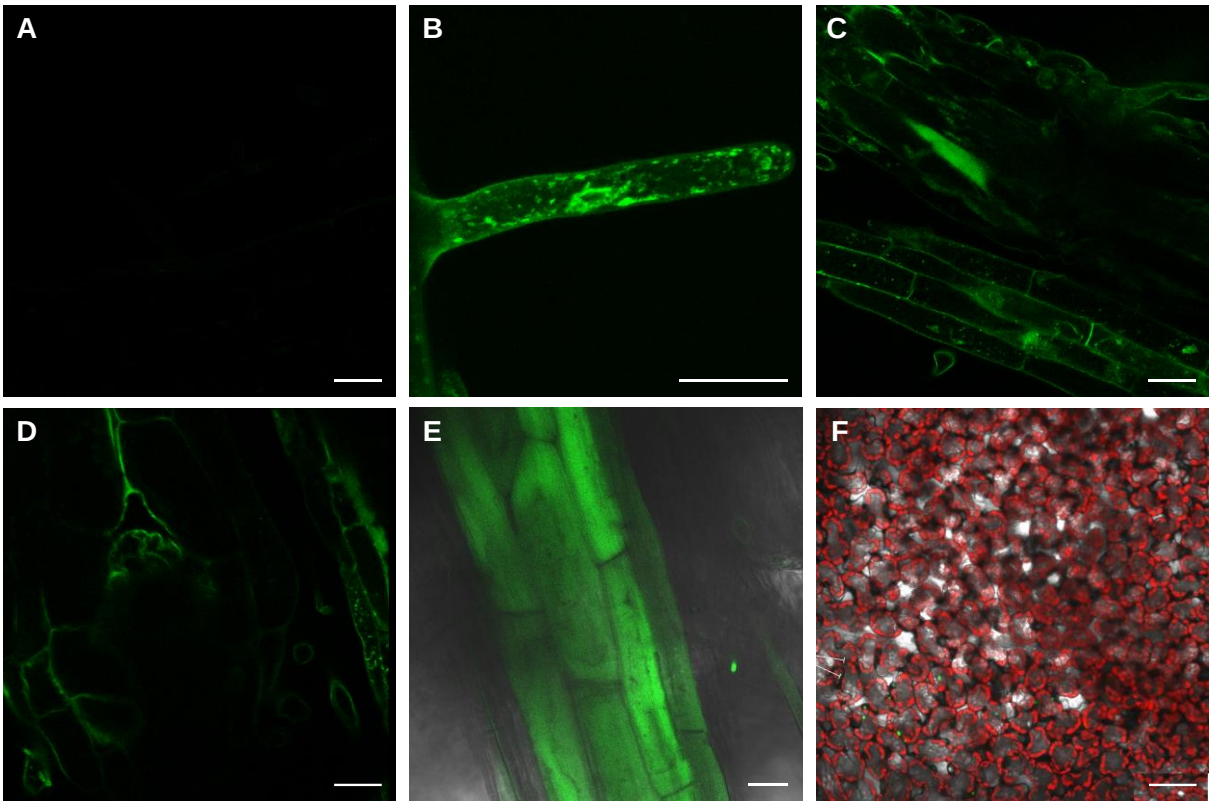

Fig. 5-7: Click-labeling and fluorescence imaging of Arabidopsis seedlings treated with a 1.5 mM TAG4 solution. All images were taken on Arabidopsis seedlings after 48 hours of incubation with TAG4 or water and a subsequent CuAAC with Alexa fluor 488 **A**: Fluorescent picture shows roots of mock-treated Arabidopsis seedlings with no labelling detected. **B**: maximum projection of a root hair of Arabidopsis seedlings showing an epidermal cell wall pattern and intracellular globular structures. **C**: fluorescent image of root and hypocotyl of Arabidopsis seedlings with an epidermal cell wall labelling and intracellular globular structures. **D**: fluorescent image of a hypocotyl of Arabidopsis seedlings showing epidermal cell wall labelling. **E**: fluorescent image of Arabidopsis seedlings root cells showing a diffuse labelling. **F**: merged image of Arabidopsis seedlings leaves without labeling detected. Scale bar correspond to 25 μm.

5.3.3. Alkyne-tagged-BABA analogues and Proline-TAG5 show the same pattern of localization as TAG4

To further investigate whether this pattern might be due to the chemical modifications introduced in TAG4, we firstly compared it with TAG5, as the structures of the two TAGs are similar and only differ from an ether group in the added function (**Fig. 5-2**). Interestingly, TAG5 and TAG4 share cell wall localization and dot-like globular structures in epidermal cells (**Fig. 5-8.A**). Moreover, in roots of TAG5 treated-Arabidopsis spots were seen to float following cyclosis (**Video S5**). Interestingly, TAG5 does not localize in emerging secondary root buds **Fig. S5-2.A and .B**) and, as occurring for TAG4, neither in leaves (**Fig. 5-8.B**).

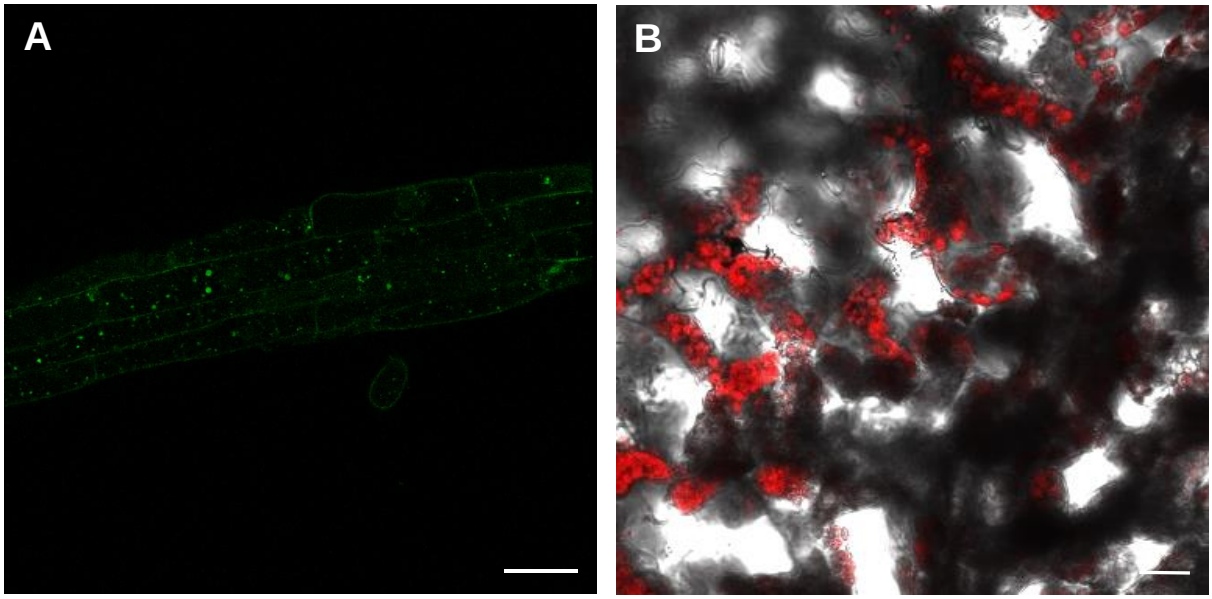

Fig. 5-8: Click-labeling and fluorescence imaging of Arabidopsis seedlings treated with a 150 µM TAG5 solution. A: fluorescent picture representing roots and root hairs of Arabidopsis seedlings incubated with TAG5 48 hours post-treatment and CuAAC with Alexa fluor 488. Labelling localize in cell wall of epidermidis and root hairs. Intracellular globular structures also have been detected. **B**: merged picture shows leaves of Arabidopsis seedlings incubated 48 hours with TAG5 with no labeling detected after CuAAC with Alexa fluor 488. Scale bar correspond to 25 µm.

Successively, we wanted to confirm whether the TAG4 and TAG5 pattern of localization was dependent was also present treating plants with the other alkyne-modified BABA. Therefore, we treated Arabidopsis seedlings with 150 µM solutions of TAG1 and TAG6 as well. Mock-treated plants did not show any labelling, while in TAGs-treated plants share the same epidermal roots and root-hairs-cell wall localization pattern found in TAG4 and TAG5-treated plants (**Fig. S5-3**Errore. L'origine riferimento non è stata trovata.). Absence of a specific labelling in leaves in all the TAGs treated plants has been shown as well (**Fig. S5-3**). However, no globular structures have been seen in the TAG1 and TAG6 treatment, compared to TAG5 and TAG4 (**Fig. S5-4**).

Finally, to confirm if this pattern was specific of alkyne-modified BABA or this could be shared with other alkyne-modified amino acids, we treated Arabidopsis seedling with a solution of 150 µM of proline-TAG5. Interestingly, proline-TAG5 localized in epidermal root cell wall and dot-like structures also were detected (**Fig. S5-4.A**). Furthermore, no labelling was found in leaves, similarly as occurring after BABA-TAGs treatments (**Fig. S5-4.B**). These results show that alkyne-modified amino acids show the same patterns of localization, accumulating in root cell walls and also in globular intracellular structures, but not in the aerial part of the plant.

5.4. Discussion

The subcellular localization of BABA is one of the major challenges in the knowledge of this metabolite in plants. First attempts to elucidate this aspect were conducted treating different plant species with ^{14}C-BABA solutions, spraying or soil drench (Cohen and Gisi, 1994; Cohen et al., 1999; Jakab et al., 2001; Cohen et al., 2010). These works demonstrated that BABA moves in plants basipetally and acropoetally and localizes preferably in younger tissues after treatment, where activates the defense response (Cohen and Gisi, 1994; Cohen et al., 1999; Jakab et al., 2001; Cohen et al., 2010). However, if Cohen and Gisi (1994) and Cohen et al. (2010) found a retention of ^{14}C-BABA in the cell wall fraction, Jakab et al. (2001) found only a cytoplasmic accumulation. Later, the work of Balmer et al. (2019) demonstrated that plant-produced BABA localizes mainly in seeds, flowers and siliques. Furthermore, it was demonstrated that plants accumulate BABA in locally infected leaves after both *P. cucumerina* and *Pst* AvrRpt2 infection but not in systemic leaves nor in roots (Balmer et al., 2019). Unfortunately, no data for subcellular localization of endogenous BABA are not known yet.

This work tried to clarify the different the subcellular localization of BABA in plants using CuAAC. CuAAC has emerged as a new tool to understand the glycobiology of plant cell wall (Anderson et al., 2012; McClosky et al., 2016; Zhu et al., 2016). For example, incubating Arabidopsis seedlings with N-azidoacetylglucosamine and performing successively CuAAC, Zhu et al. (2016) found incorporation of the azide-modified sugar into the N-linked glycans and a localization in the elongation zone of the root. Another work demonstrated that fucose localized in the inner layer of the cell wall adjacent to the plasma membrane using as an alkyne-modified fucose (Anderson et al., 2012). Our results showed a localization of TAG4 in the cell wall of root epidermidis of Arabidopsis seedlings. TAG4 localization was also shown in the hypocotyle cell walls when applied at higher concentrations. Therefore, BABA seemed to be retained in the cell wall as already shown in Cohen and Gisi (1994). Interestingly, the pattern of localization of TAG4 resembles that of of alkyne-fucose and N-azidoacetylglucosamine (**Fig. 5-9**) (Anderson et al., 2012; Zhu et al., 2016). Interestingly, 1.5 mM TAG4 application showed also a diffuse pattern, although TAG4 did not localize in the interstices between cells (**Fig. 5-9**). Nevertheless, it is difficult with only CuAAC to clarify the nature of the diffuse pattern. However, alkyne-fucose treatment produced a similar diffuse pattern in newly elongated root cells (**Fig. 5-9**) (Anderson et al., 2012). Furthermore, alkyne-fucose-Alexa complex was found to be highly localized in root hairs, similarly as occurring incubating

Arabidopsis seedlings with TAG4 (**Fig. 5-9**). Nevertheless, although similar to the cell wall patterns of alkyne-modified sugars, other experiments are required to explain the cell wall localization of BABA in plants. For example, it is worthy important to understand the developmental site where BABA localizes in roots and understand why secondary root buds do not accumulate TAGs.

Moreover, TAG4 localized inintracellular globular structures. Similar structures have been already described after CuAAC between alkyne-fucose and Alexa-488 azide (**Fig. 5-9**), where are found mainly in the early differentiation zone (Anderson et al., 2012). The globular pattern found for TAG4 was shared with TAG5 and Proline-TAG5 as well (**Fig. S5-4**), but not with TAG1 and TAG6. These TAGs were modified in the methyl group, supposing that this group is important for the localization of BABA and other amino acids in globular structures. Globular structures may represent the fusion sites of Golgi-derived alkyne-fucose containing vesicles and the plasmalemma, as supposed by Anderson et al. (2012). Nevertheless, the movement following the cytoplasmic stream of the globular structures may let think to an artifact due to the CuAAC. However, the alkaline conditions of the buffer for CuAAC could kill the plant and alter intracellular movements, letting open the possibility of a vesicular localization of BABA in Arabidopsis roots.

According to the data shown in this work, BABA may be incorporated into structural cell wall proteins, possibly by crosslinking as hypothesized by Cohen and Gisi (1994). However, Cohen and Gisi (1994) detected ^{14}C-BABA in the cell wall fraction of tomato leaves extracts, as well as Jakab et al. (2001) in Arabidopsis, although with a different organellar localization. Our results did not show TAG4 localization in leaves. Neither the other TAGs nor Proline-TAG5 localize in leaves. Nevertheless, TAGs application is able to induce resistance against *Hpa* NOCO, reducing conidiospore production. Furthermore, TAG4 is able to induce resistance against *P. cucumerina* and *Pst* DC3000. This resistance capacity, although less effective than BABA, was measured in leaves after TAG-soil drench treatment, supposing that TAGs translocate acropetally to activate immunity. Nevertheless, Cohen et al. (2010) demonstrate that N-modified BABA molecules (DL)-3-Methylamino-butyric acid and (DL)-3-Benzylamino-butyric acid do not induce resistance against *B. lettuce* in lettuce plants. An explanation may be found in a cleavage of the bonds with the alkyne group of TAGs in roots, releasing free BABA that translocate in the aerial part of the plant inducing resistance.

In conclusion, these results although pioneering, are not sufficient to clearly define the subcellular localization of BABA and further experiments are required to confirm our data.

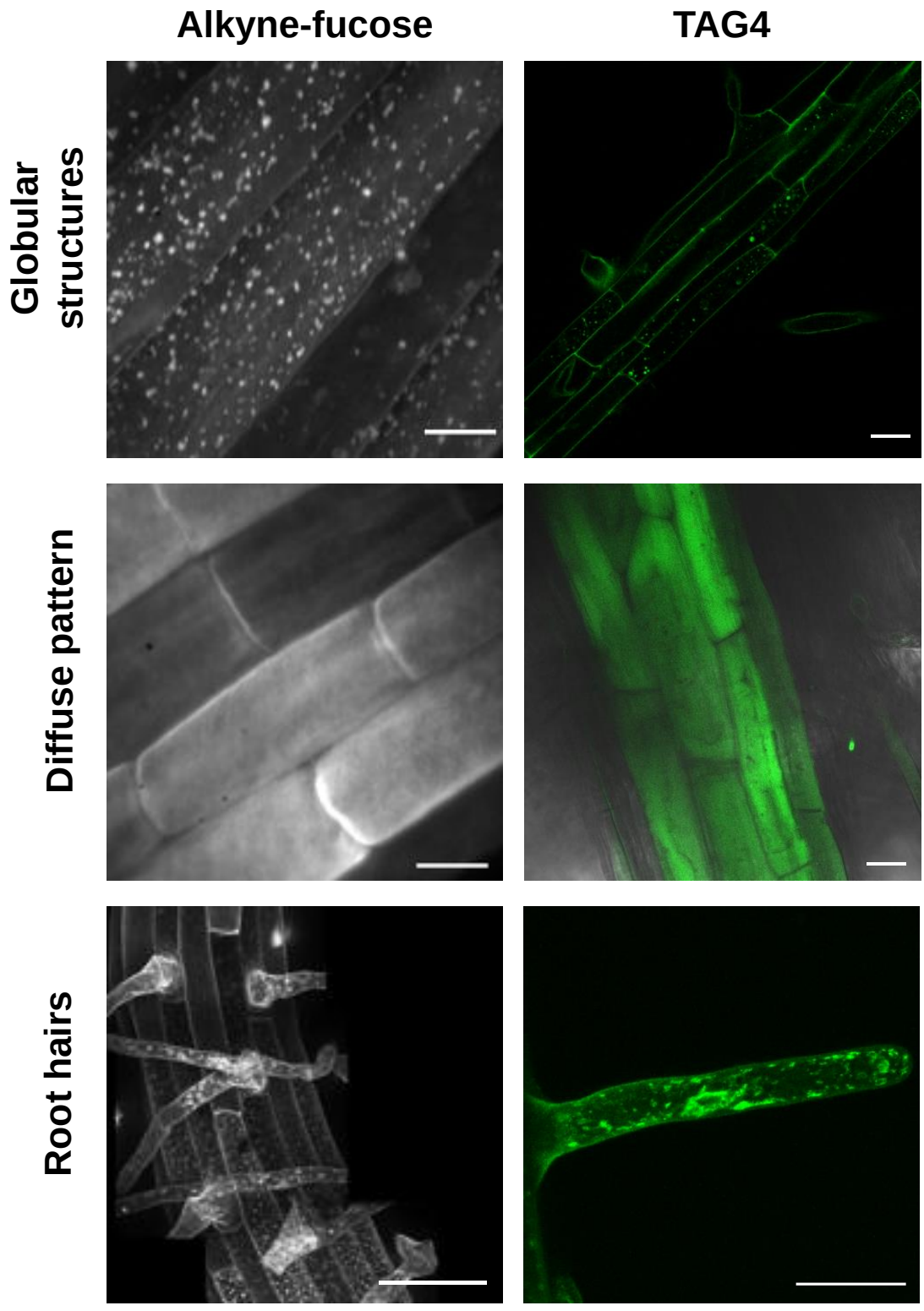

Fig. 5-9: Similar patterns between Alkyne-fucose and TAG4. Left column: Four-day-old Arabidopsis Col-0 were incubated for 4 hours with 2.5 µM of Alkyne-Fucose and then treated with a 0.1 µM Alexa fluor 488 azide solution. All the images concerning Alkyne-Fucose are at maximum projection, while scale bar in the "globular structure" and "diffuse pattern" row pictures represent 10 µm, while for "root hairs" 50 µm. Images taken from Anderson et al. (2012). **Right column:** different images taken from this work representing TAG4-Alexa 488 complex localization pattern. White bar represents 25 µm.

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5.6. Supplementary material

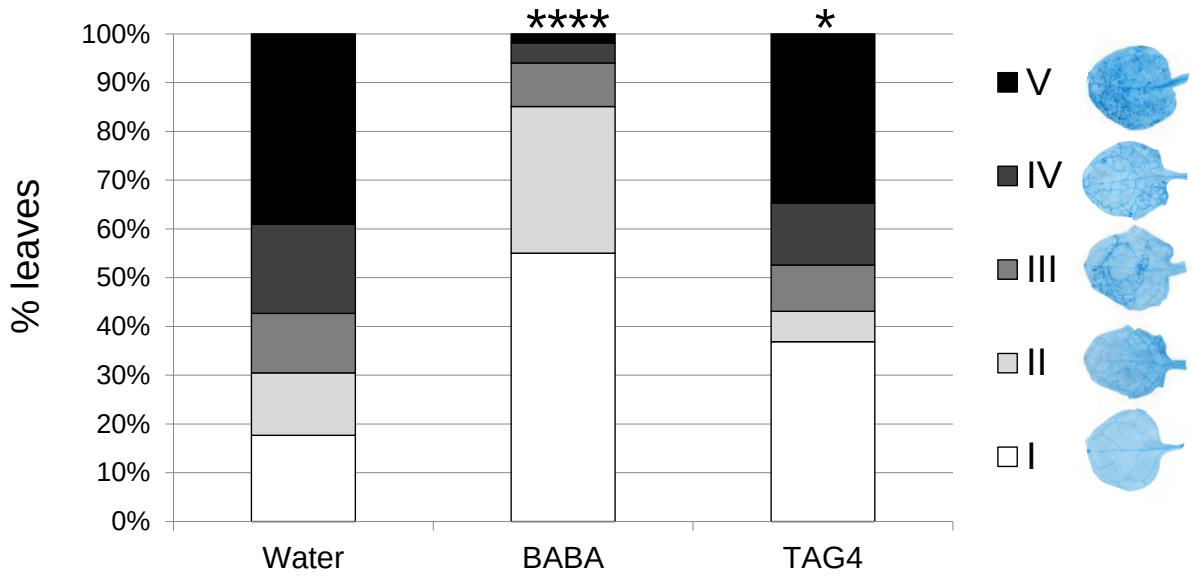

Fig. S5-1: TAG4 resistance inducing capacity against *Hyaloperonospora arabidopsidis* NOCO. Fifteen two-weeks-old BABA and TAG4-treated Arabidopsis Col-0 seedlings have been inoculated with *Hpa* Noco. Disease severity was quantified at 7 days after inoculation. Disease-severity rating is presented as the percentages of leaves in disease. Classes I (no sporulation), II (less than 50% of the leaf area covered by sporangia), III (more than 50% of the leaf area covered by sporangia), and IV (completely covered with sporangia, with additional chlorosis and leaf collapse). Asterisks indicate significant differences as determined by a χ^2 test comparing treated and mock-treated plants: *P < 0.05; ****P < 0.001.

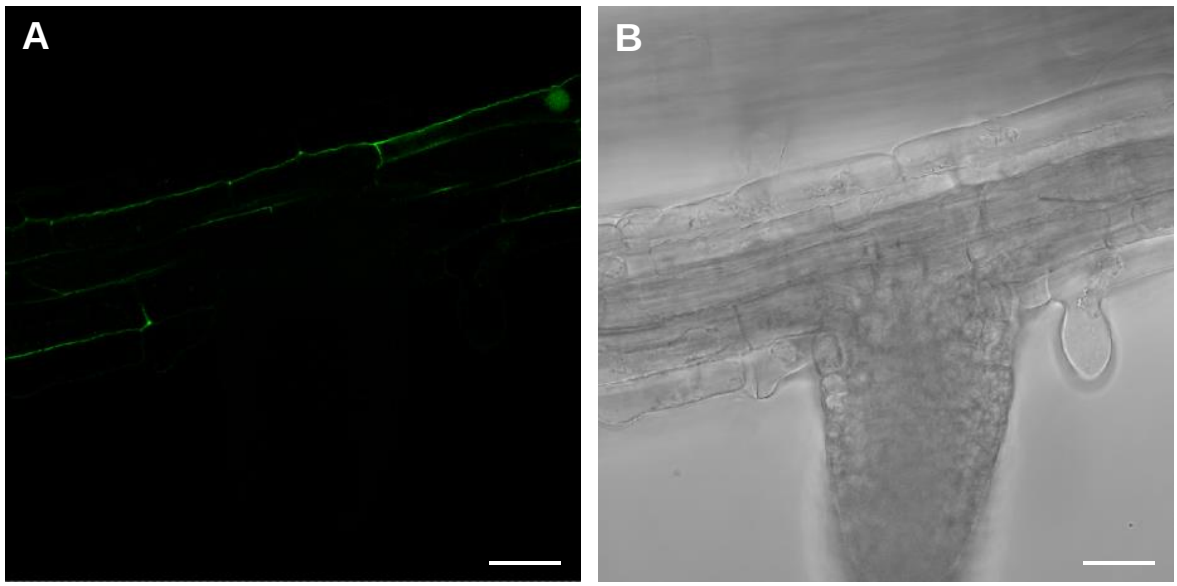

Fig. S5-2: Click-labeling and fluorescence imaging of Arabidopsis seedlings treated with a 150 µM TAG5 solution. A: fluorescent picture representing roots and secondary root bud of Arabidopsis seedlings incubated with TAG5 48 hours post-treatment and CuAAC with Alexa fluor 488. Labelling localize in cell wall of epidermidis of the main root but not in the emerging secondary root. **B**: bright field picture of the same sample used for picture A. Scale bar correspond to 25 µm.

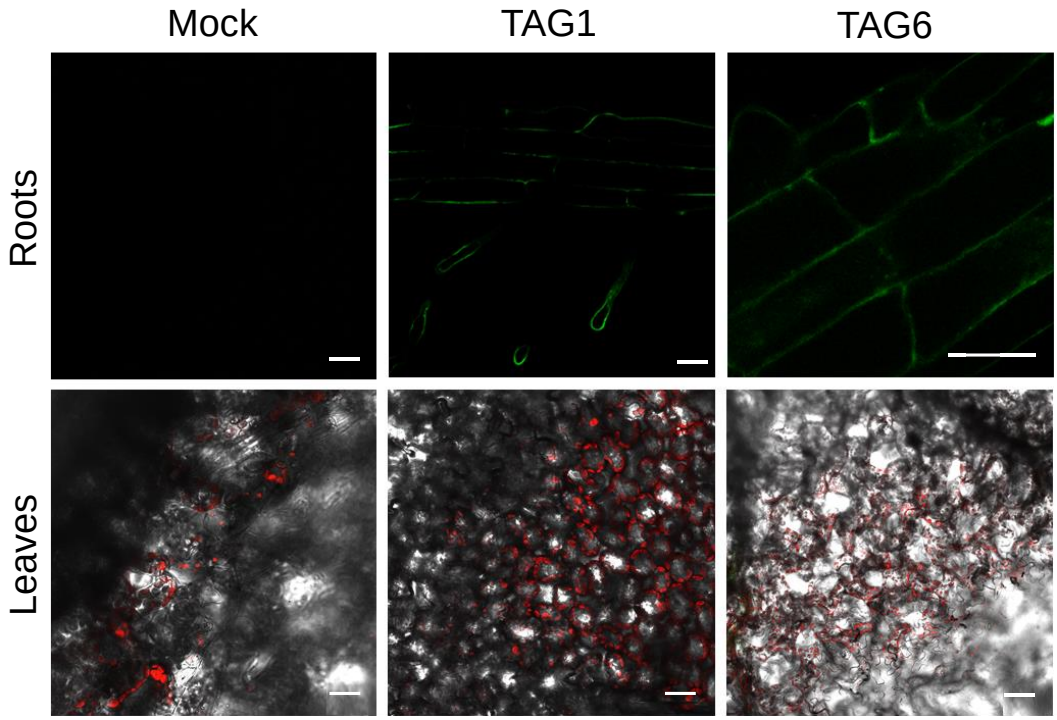

Fig. S5-3: Click-labeling and fluorescence imaging of Arabidopsis seedlings treated with 150 µM TAG1 and TAG6 solutions. Fluorescent pictures represent roots of Arabidopsis seedlings incubated with TAG1 and TAG6 48 hours post-treatment and CuAAC with Alexa fluor 488. Labelling localize in cell wall of epidermal cells in both treatments. Merged pictures represent leaves of TAG1 and TAG6-treated seedlings after CuAAC with Alexa fluor 488, with no labeling detected. Scale bar correspond to 25 µm

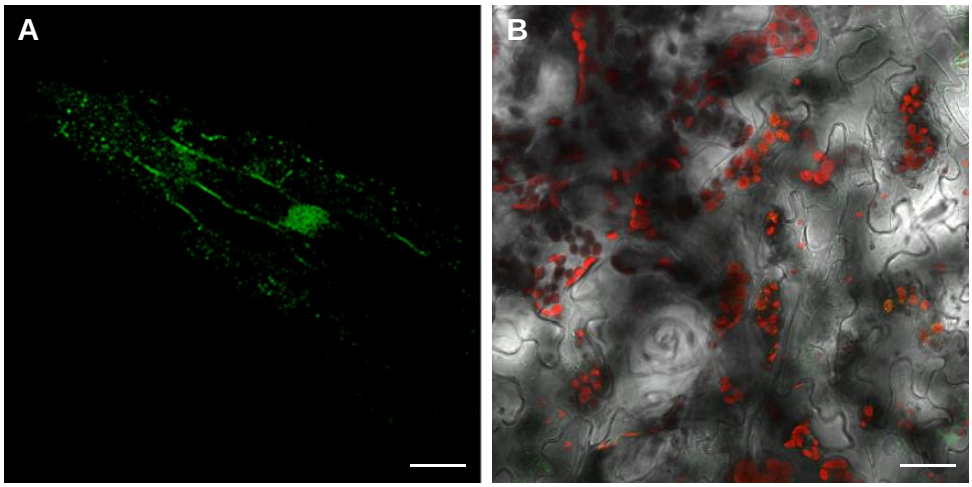

Fig. S5-4: Click-labeling and fluorescence imaging of Arabidopsis seedlings treated with a 150 µM proline-TAG5 solution. A: fluorescent picture representing root of Arabidopsis seedlings incubated with proline-TAG5 48 hours post-treatment and CuAAC with Alexa fluor 488. Labelling localize in cell wall of epidermal cell and intracellular globular structures have been detected as well. **B**: merged picture represents leaves of the same seedling of picture A, with no labeling detected. Scale bar correspond to 25 µm

Supplementary video: BABA-TAG5 globular structures movements.mp4

6. Conclusions and perspectives

Francesco Stefanelli

6.1. Searching genes involved in BABA metabolism in Arabidopsis

The project for searching candidate genes involved in BABA metabolism in Arabidopsis using RNA-seq analysis did not reach the goal it was set-up for. Several aspects of the methodology employed might have affected the results. For example, T-DNA insertional lines of the genes found with RNA-seq other than those employed up to now have to be checked. Indeed, the lines we used in this work may not display an impaired BABA accumulation phenotype because plants would still express transcripts. Therefore, it is important to look at the transcripts accumulation in each line by qRT-PCR. In addition, we could look at BABA concentrations after salt stress application at an earlier time point than that used, to check whether BABA accumulation starts later than wild type in the different mutants.

Furthermore, we assumed that transcripts of BABA-metabolic genes follow the "signal delay" model of the dependency of protein level and mRNA accumulation (Liu et al., 2016). Nevertheless, transcripts may follow the "translation on demand" model as well (Liu et al., 2016), where the mRNA levels keep stable while translation increases after a stimulus. If BABA-related genes are transcribed and translated according to this model, a transcriptomic approach may not be the best way to find such genes. Therefore, proteomic analysis using LC-MS/MS may be a better method to find genes involved in BABA metabolism. For the set-up of a proteomic approach would be important to time frame of highest production of BABA, looking at the trend of biosynthesis during time during different stresses. Afterwards, time points for protein extraction would be chosen when the curve presents an exponential trend. The comparison between the proteomics of different BABA-inducing stresses and *cpr5-2* may find genes involved in BABA biosynthesis and maybe BABA-interacting proteins as well. This would be a good way to find whether *IBI1*, the receptor of exogenously applied BABA (Luna et al., 2014), may work as receptor for endogenous BABA as well. Furthermore, comparing the proteomics of stressed-plants when the BABA-concentration curve has a descendent trend may help to find candidate genes involved in BABA catabolism.

Another interesting approach to find genes involved in BABA metabolism in Arabidopsis may be genome wide association (GWA) study. GWA correlates a genetic marker, *e.g.* single nucleotide polymorphisms (SNPs), and a measurable trait among the individuals of a

population. This correlation may allow finding candidate genes associated with phenotype analyzed. (Brachi et al., 2011; Assmann, 2013; Proietti et al., 2018). GWA is a genetic approach firstly applied in humans (Hirschhorn and Daly, 2005), but then found a wide application in plant science as well (Aranzana et al., 2005; Assmann, 2013; Proietti et al., 2018). Proietti et al. (2018), for example, employed GWA for searching genes involved in the JA-SA and JA-ABA crosstalk. Firstly, authors looked at the genetic variations in the expression of the JA-response gene PDF1.2 after SA and ABA treatment in multiple Arabidopsis accessions (Proietti et al., 2018). Secondly, using GWA mapping resource of the Arabidopsis haplotype map collection, Proietti et al. (2018) found several interesting loci associated with SA and ABA crosstalk with MeJA. Thirdly, they used T-DNA insertional lines of the candidate genes found with GWA for their resistance capacity against *B. cinerea* and the herbivore *Mamestra brassicae* (Proietti et al., 2018). Therefore, a similar experiment could be set-up for searching genes involved in BABA biosynthesis and regulation. Firstly, BABA concentrations would be analyzed in several Arabidopsis accessions during stressed and un-stressed conditions. Afterwards, GWA mapping may reveal different loci associated with BABA accumulation, and the candidate genes may be tested for their capacity in synthesizing and accumulating BABA using T-DNA insertional lines.

6.2. Dissecting the relation between the novel plant metabolite BABA and phytohormones, focusing particularly on ABA-signaling.

In this project, we found that the racemic ABA induced BABA when soil-drench applied. The bioactive form of ABA (+)-ABA was also able to increase BABA concentrations when sprayed on Arabidopsis Col-0 leaves. Nevertheless, the other hormones applied, such as MeJA, SA, ET precursor ACC, Kinetine, IAA and GA3, did not induce BABA neither sprayed nor injected in soil. Furthermore, we found that in ABA-signaling mutants *snrk2.2 snrk2.3* and *ost1/snrk2.6*, (+)-ABA treatment increased BABA concentrations. However, *snrk2.2 snrk2.3* double mutant showed a slighter induction compared to *ost1/snrk2.6*, suggesting a possible role in regulating BABA accumulation after ABA application.

To have a clear view of the relation between BABA and plant hormones it is important to look for each hormone in a larger time frame and not only at a single time point, as each

hormone may induce BABA with a different trend. Furthermore, it would be interesting to look at BABA concentration after hormonal application not only in control conditions, but also during stresses, both biotic and abiotic. Furthermore, it would be interesting to look at BABA concentrations after (-)-ABA application, to definitely confirm that is only the active form of ABA, (+)-ABA, able to induce BABA.

Afterwards, considering the role of ABA in the physiology of BABA in plants, it would be interesting to look at BABA concentrations after ABA treatment in a *snrk2s* triple mutant. The triple mutant we used, *snrk2.2 snk2.3 snrk2.6* used in Fujii and Zhu (2009), did not grow enough for extracting BABA. Therefore, we could not establish a precise correlation between ABA-dependent BABA induction and ABA signaling in Arabidopsis. However, other allele of the three *SnRK2* genes, for example *snrk2d, snrk2e* and *snrk2i* and corresponding double and triple mutants (Fujita et al., 2009) may give additional information regarding the relation between ABA and BABA. These mutants show similar ABA-insensitive phenotypes as those we used (Fujii et al., 2007; Fujii and Zhu, 2009; Fujita et al., 2009) and are good candidates for further experiments.

In addition, it would be interesting to look at BABA concentrations during different stressful conditions in ABA-signaling and ABA-biosynthetic mutants. Particular attention may be given on osmotic stress, as ABA is a key regulator on the response to this stress. In fact, OST1/SnRK2.6 is the SnRK2 regulating ABA response during osmotic stress, notably stomatal closure (Mustilli et al., 2002), and it would be interesting to look at BABA concentrations during this stress. The ABA-deficient mutant *aba2-1* accumulates 10 times less ABA than wild type and does not increase ABA concentrations during osmotic stress (Léon-Kloosterziel et al., 1996). Therefore, it could be used for experiments concerning a possible role of ABA in BABA accumulation during salt-stress. Furthermore, the role of ABA in BABA accumulation during senescence may be explored using the *snrk2s* mutants we employed in this work. Firstly, it would be interesting to look at BABA concentrations during the different ages of plants. Secondly, BABA concentrations can be detected in detached leaves where senescence is induced in dark conditions, as described in Balmer et al. (2019).

ABA-independent signaling mutant, such as *dreb2a* (Liu et al., 1998; Sakuma et al., 2006a; Sakuma et al., 2006b; Vainonen et al., 2012), may be employed whether ABA would not be involved in BABA accumulation during salt stress. For this reason, it would be interesting also to explore the role of NO, another player in the response to osmotic stress, in BABA accumulation during salt stress and other stressful conditions. For this study, good candidates

are the Arabidopsis NO-non accumulating mutants *nia1-1 nia2-5/chl3-5* (Wilkinson and Crawford, 1993) and *atnoa1* (Guo et al., 2003; Zeidler et al., 2004; Domingos et al., 2015). *nia1 nia2-5/chl3-5* shows decreased NO synthesis and *NIA1* and *NIA2* play a role in the NO-dependent stomatal closure (Bright et al., 2006; Modolo et al., 2006; Domingos et al., 2015). On the other side, AtNOA1 is a mitochondrial-targeted protein firstly though to be bona-fide a NO-synthase (Guo et al., 2003). Up-to now, AtNOA1 is no more considered as NO-synthase, but is one of the few mutants not accumulating NO, and is widely used as tool for studying NO physiology in Arabidopsis (Domingos et al., 2015).

6.3. BABA subcellular localization

Our results showed an interesting root cell wall localization pattern for alkyne-modified BABA molecules, suggesting that BABA could localize in cell wall by cross-linking cell wall proteins. However, in contrast with previous papers (Cohen and Gisi, 1994; Jakab et al., 2001), our results did not show a localization of TAGs in leaves. However, soil drenched TAGs were able to induce a slight resistance against different pathogens in leaves, but this induction may be due to the release of free BABA. Therefore, other experiments have to be performed to elucidate the subcellular localization of BABA.

Firstly, to confirm if the cell wall is the site of BABA accumulation in roots, a co-application of TAGs and Potamine Fast Scarlet 4B, a fluorescent label for cellulose (Anderson et al., 2010), could be achieved. Secondly, to test whether the globular structures belong to the vesicular trafficking, co-localization with a fluorescent marker for Golgi, vesicles and reticulum could be performed. Different fluorescent lines of organel markers exists Generally, CuAAC quenches fluorescents-labeled proteins. However, Bourge et al. (2015) re-obtained florescence from GFP washing with EDTA CuAAC-treated plants in order to keep out copper for proteins and visualizing them at a neutral pH. This method led to the contemporary visualization of both alkyne-modified 5-ethynyl-20-deoxyuridine, a timidine analogue, and the GFP-labeled Golgi marker Sialyl transferase (ST)-Kaede (**Fig. 6-1.A**) (Bourge et al., 2015).

Other techniques exist to localize small molecules in plant tissues using microscopy. Mass Spectrometry Imaging (MSI), for example, has been used to visualize small molecules, such as lipids, proteins and secondary metabolites, in different plant tissues (Lee et al., 2012). Matrix-assisted laser desorption/ionization (MALDI) combined with MSI allows to detect also the

cellular and even subcellular localization of small molecules (Sturtevant et al., 2016). Although with a lower resolution than other microscopical techniques, MALDI-MSI can detect contemporary hundreds of analytes, does not employ label and can detect unknown molecules as well, as it combines metabolomics with microscopy (Buchberger et al., 2018). A typical MSI experiment workflow starts with sample slice collection, followed by the embedding of the sample in a supporting medium and placing it onto slides (Buchberger et al., 2018). Successively, a matrix is added to the sample whether a MALDI-MSI is employed. Afterwards, a laser scans the different (x,y) grid points on the sample ionizing the molecules and producing thus a spectrum (Buchberger et al., 2018). A software acquires the data and produces an image of the distribution of the different molecules in the sample, which are identified by their mass/charge (m/z) ratio (Buchberger et al., 2018). O'Neill and Lee (2020) employed MALDI-MSI for detecting differences in free amino acids composition of roots of different maize cutivars. Nevertheless, amino acids are difficult to detect with MALDI-MSI. Therefore, O'Neill and Lee (2020) derivatized amino acids with conifer aldehyde for increasing the ionization efficiency. This technique has been also used for the study of primary amines (Seebauer et al., 2004). Interestingly, among the different amino acids observed, they could visualize GABA as well, finding a localization pattern in the parenchyma of the root (**Fig. 6-1.B**) (O'Neill and Lee, 2020). Consequently, MALDI-MSI appears as s good alternative to study the localization of BABA in plants. Furthermore, MALDI-MSI may give additional information not only on exogenously applied BABA but on plant-produced BABA as well, giving the possible first clues for the localization pattern of this amino acid in plants.

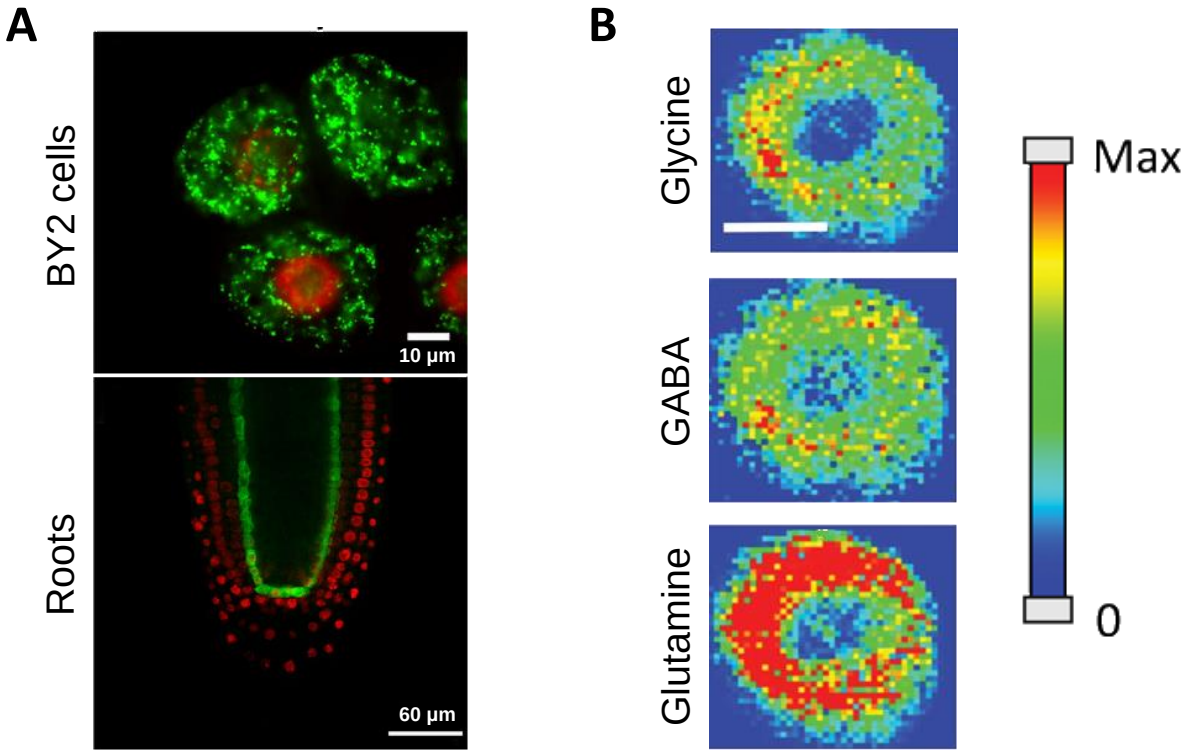

Fig. 6-1: Co-localization of alkyine-modified EdU and GFP-tagged ST-Kaede, and amino acids localization with MALDI-MSI. (A) GFP-ST-Kaede transformed *Nicotiana tabacum* BY2 cells and roots of Arabidopsis thaliana treated with alkyne-EdU. Image from Bourge et al. (2015) **(B)** MALDI-MS images of Glycine, GABA and Glutamine in maize roots. Colours represent the maximum intensity scale with dark blue as 0 and maximum as red. Scale bar represent 500 µm. Image from O'Neill and Lee (2020).

6.4. References

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