

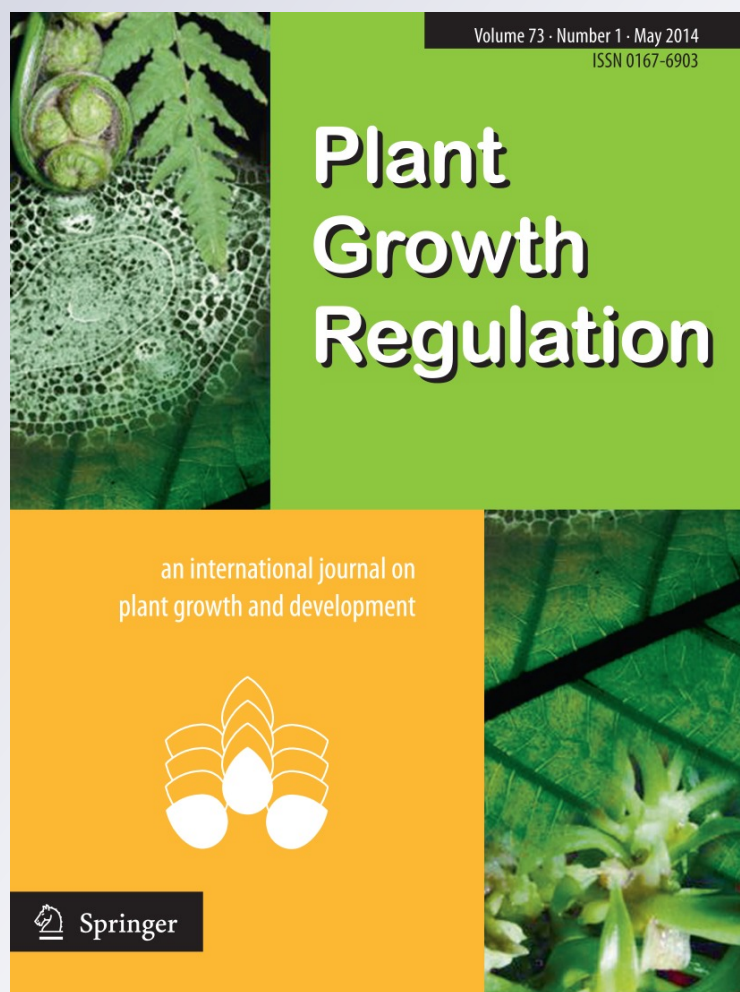
Influence of 24-epibrassinolide on lipid signalling and metabolism in Brassica napus

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Influence of 24-epibrassinolide on lipid signalling and metabolism in *Brassica napus*

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Abstract Due to the increasing demand for biofuel production, it is an important goal to optimize the seed productivity and quality of oilseed plants even in adverse conditions. Acting on signalling mechanisms might provide means to attain such goals. In this study, we were interested in the effect of a brassinosteroid hormone 24-epibrassinolide (24-EBR) on *Brassica napus* cultivated in salt stress condition. We show that salt stress leads to a 60 % decrease in seed production in *B. napus*. This is accompanied by a 50 % decrease in seed oil content. Treatment with 24-EBR had no effect on seed and oil productivity in control plants. However, it could rescue half of the seed production and all the oil production in salt-treated plants. The fatty acid composition of seed oil in *B. napus* was selectively affected by salt stress, 24-EBR or combined treatment. Besides these

long-term actions of 24-EBR, we have also investigated its short-term actions in cell signalling. We did so by in vivo labelling of plantlets with fluorescently labelled phosphatidylcholine. A treatment of 2 h with 24-EBR was sufficient to induce a substantial increase in the content of diacylglycerol and phosphatidic acid, two lipid mediators. Non-specific phospholipases C and phospholipases D are involved in these increases. Therefore, brassinosteroid treatments appear as promising way to gain oil productivity when plants have to grow in unfavourable conditions such as salt stress. The link between long-term actions and short-term signalling of 24-EBR is discussed.

Keywords 24-Epibrassinolide · Oil productivity · Salt stress · Fatty acids · Phospholipase D · Non-specific phospholipase C

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Introduction

Plant oil is now considered as one of the main sources of biorenewable fuels. In the majority of plants, oil is stored in seeds as triacylglycerols (TAG) that will serve as an energy reserve required for the initial growth of seedlings in the absence of established photosynthesis. In plants, TAG biosynthesis pathway is known to be complex (Fig. 1). All fatty acids (FA) initially originate from a pool of acetyl-coenzyme A (CoA) that is located in the plastids. Then, plastid-localized and endoplasmic reticulum-localized enzymes participate in the biosynthesis of all main cell glycerolipids, including storage TAGs (Voelker and Kinney 2001; Chapman and Ohlrogge 2012). Glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidic-acid acyltransferase (LPAAT) and diacylglycerol acyltransferase (DGAT), that catalyse the consecutive acylation steps of the

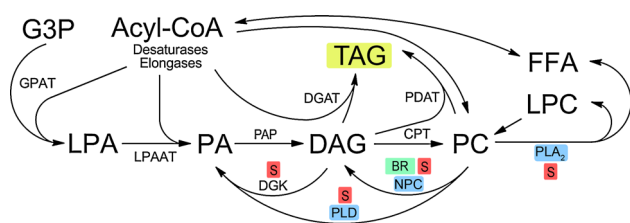


Fig. 1 Simplified scheme of plant TAG biosynthesis pathways. Metabolic steps affected by salt stress (S), brassinosteroids (BR) or controlled by phospholipase activity are highlighted (Kocourková et al. 2011; Wimalasekera et al. 2010; Pokotylo et al. 2012). DAG, diacylglycerol; DGAT, DAG acyltransferase; DGK, diacylglycerol kinase; G3P, glycerol 3-phosphate; FFA, free fatty acids; G3P, glycerol 3-phosphate; GPAT, glycerol-3-phosphate acyltransferase; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; LPAAT, lysophosphatidic acid acyltransferase; PA, phosphatidic acid; PAP, phosphatidic acid phosphohydrolase; PC, phosphatidylcholine; PLD, phospholipase D; NPC, non-specific phospholipase C; PLA₂, phospholipase A₂; TAG, triacylglycerol; CPT, choline phosphotransferase; PDAT, phospholipid:diacylglycerol acyltransferase

glycerol-3-phosphate backbone, are all critical for TAG biosynthesis. Alternatively, the *sn*-3 acylation of DAG can be achieved by the combined actions of plant-specific choline phosphotransferases (CPT) and phospholipid:diacylglycerol acyltransferases (PDAT). Most of these pathways have been characterized both at biochemical and molecular levels (Beisson et al. 2003). Yet, how plants achieve an efficient metabolic biosynthesis in adverse conditions is not entirely understood.

Nevertheless, there have been numbers of attempts to develop crop plants with improved oil yield or providing oils with specific FA composition. Several biotechnological approaches were implemented including genetic engineering aimed at manipulating the lipid/FA biosynthesis pathways (Tan et al. 2011; Ruiz-López et al. 2012; Bates and Browse 2012). However, it is becoming clearer and clearer that oil productivity is a complex trait controlled by several enzymatic complexes (Tang et al. 2012). That is why affecting individual enzymes in lipid biosynthesis pathways may not be as effective as originally expected (Ohlrogge and Jaworski 1997). In contrast, treatments of plant with hormones, that orchestrate plant development and influence major metabolic fluxes, might be a promising way of modulating oil quantity and quality in seeds. Indeed, synthetic cytokinins were shown to affect oil accumulation in *Lavandula dentata* plantlets (Sudriá et al. 2001) and rhizobacteria-derived auxins increased the oil quantity in *Brassica juncea* (Asghar et al. 2002). Brassinosteroids (BRs) constitute a unique group of plant steroid growth-promoting hormones that, at low concentrations, act as important metabolism regulators (Kim and Wang 2010). BRs, that include 24-epibrassinolide (24-EBR), are sensed by BRASSINOSTEROID-INSENSITIVE 1 receptor kinase and activate distinct signal transduction cascade

and cellular responses (Clouse 2011). Receptor-independent effects of lipophilic BRs on plant metabolism and plant membranes have also been suggested (Albrecht et al. 2011; Zhang et al. 2005). BRs stimulate plant growth and photosynthesis (Xia et al. 2009) and affect activity of antioxidant systems (Zhang et al. 2010). BRs can also increase plant stress tolerance (Divi et al. 2010) and improve *B. napus* germination and growth in drought and saline conditions (Kagale et al. 2007). The role of BRs in stomata development has also been demonstrated (Kim et al. 2012) and may be crucial for water-use efficiency in salt-stressed plants. However, the exact mechanisms of BR-induced plant regulation are unclear.

The intracellular action of BRs may be functionally associated with phospholipases that control many plant growth reactions (Wang et al. 2012). Phospholipases hydrolyse membrane lipids to produce second messengers essential for regulatory signalling. Phospholipases also contribute to generic lipid metabolism and lipid turnover (Li et al. 2006) including TAG biosynthesis (Lee et al. 2011). In addition, phospholipases are well known to participate in stress and hormone signalling (Janda et al. 2013). As an example, phosphatidic acid (PA) production via phospholipase D activity was rapidly induced in salt-stressed tobacco (Pokotylo et al. 2012) while overexpression of phospholipase D α resulted in improved drought and salt tolerance of plants (Peng et al. 2010; Zhang et al. 2008). Interestingly, plant non-specific phospholipases C (NPC), which are able to directly cleave structural membrane phospholipids to produce diacylglycerol (DAG) (Pokotylo et al. 2013), were activated after BR treatment in tobacco BY-2 cells (Wimalasekera et al. 2010).

Therefore, the aim of this study was to investigate the influence of 24-EBR on oil productivity and fatty acid composition of seed oil from *B. napus* plants subjected to salt stress. The role of phospholipases in mediating BR effects is also analysed.

Materials and methods

Plant material and treatments

Brassica napus cv. Magnat plants and *Arabidopsis thaliana* Col-1 plants were used in this study. Seeds were imbibed and cold treated at 4 °C for 4 days before germination and growth. *B. napus* plants used in cultivation experiments were grown open-air in isolated vessels in alfisol-type soil subjected to temperate climate conditions with controlled watering. *Arabidopsis* plants were grown in soil under long day conditions (16 h light 125 $\mu\text{E m}^{-2} \text{s}^{-1}$; 8 h dark) at 23 °C. Salt stress was induced by watering with 0.5 M NaCl solution to achieve final concentration of 50 mM

NaCl in the soil. Chemically synthesized 24-epibrassinolide was obtained from the Institute of Bioorganic Chemistry of the National academy of Sciences of Belarus (Khripach 1990). Leaves of *B. napus* plants were sprayed with 0.1 μM solution of 24-EBR twice during the course of cultivation.

Brassica napus plants used in short-term signalling experiments were grown hydroponically in half-strength MS solution. *N*-ethylmaleimide and *n*-butanol were used at final concentrations of 5 mM and 0.5 % (v/v), respectively.

Analysis of seed oil content and FA composition

Total seed oil was extracted by the addition of 10 ml of hexane to 500 mg of freshly ground seeds followed by vacuum evaporation. FA composition of lipid samples was assessed by HPLC analysis using the method described by Chaytor (1987) with modifications. In brief, lipids were saponificated with butylated hydroxytoluene in 1 M solution of KOH in methanol over a period of 1 h at 80–90 °C. The solution thus obtained was diluted with tetrahydrofuran or acetonitrile and neutralized with 1 M orthophosphoric acid. Then, 0.2 M 18-crown-6 and 0.2 M *n*-bromophenacyl bromide were added to form FA conjugates. FA separation was performed on Agilent 1100 series HPLC with UV–Vis diode array detector. Linear gradient elution with 0.05 M orthophosphoric acid and methanol on Agilent ZORBAX Eclipse XDB-C₁₈ 5 μm and Thermo Scientific Hypersil BDS, C₁₈ 3 μm reverse phase columns was used. Absorbance peaks corresponding to individual FAs were identified using standards.

Analysis of fluorescently-labelled products of phosphatidylcholine cleavage

Roots of *B. napus* plantlets were preincubated with 0.66 $\mu\text{g ml}^{-1}$ boron-dipyrromethene (BODIPY) labelled phosphatidylcholine (BODIPY-2-decanoyl-1-(O-(11-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionyl)amino)undecyl)-*sn*-glycero-3-phosphocholine, Invitrogen) for 10 min at 24 °C on an orbital shaker prior to treatment with reagents. At the end of the experiment lipids were extracted by the addition of methanol:chloroform 2:1 (v/v). Changes in the production of phospholipid-derived second messengers were monitored using a thin layer chromatography (TLC). Unmodified silica gel 60 TLC plates (Merck) were developed with upper organic phase of the ethyl acetate/isooctane/formic acid/water (12:2:3:10, v/v) mixture. Lipid spots were visualized and counted by Pharos FX (Biorad) molecular imager. Products were identified according to Pejchar et al. (2010) and using standards.

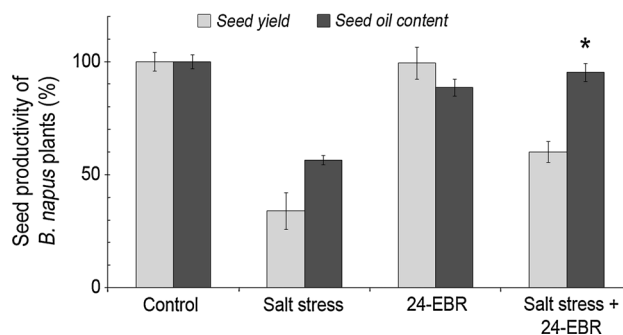


Fig. 2 Seed yield and seed oil content of *Brassica napus* cv. Magnat plants subjected to salt stress and 24-EBR. Plants were grown in soil substrate in individual containers. Salt stress was conditioned by adjusting soil NaCl concentration to 50 mM by watering. Selected plants were treated with 100 nM solution of 24-EBR by spraying during the course of germination and after 2 weeks of growth. At the end of plant vegetation, seeds were harvested, dried for 2 weeks and weighted. Seed oil was extracted from aliquot seed samples by solvents, evaporated and measured. Each seed aliquot was obtained from six plants grown in specified conditions. A statistical comparison (Student's *t* test) was performed for data obtained from salt stressed-plants and salt stressed-plants treated by 24-EBR. Data represent the mean \pm SE; *, $P < 0.05$, Student's *t* test, $n = 10$ discrete samples from two biological experiments

Results and discussion

24-Epibrassinolide application alleviates productivity loss in salt-stressed *B. napus* plants

Brassica napus are nowadays accounted to be the third most grown oil crop. We have analysed seed productivity and seed oil content of *B. napus* cv. Magnat plants under normal growth conditions and under salt stress. Plants were germinated and grown for 7 days. Then, a designated group of plants were watered with NaCl solution to achieve a final soil salt concentration of 50 mM NaCl. Seeds were collected in dry siliques and weighted. The seed oil was extracted and assayed for quantity and quality. Our results indicate that seed productivity of *B. napus* was reduced by two thirds when cultivated in salt stress conditions. Oil content of seeds obtained from plants grown in salt conditions was also significantly decreased (Fig. 2), suggesting severe inhibition of lipid metabolic pathways.

It is well known that salt stress causes an inhibition of photosynthesis, of generic metabolism and of viability (Evers et al. 2012). Though it was shown that salt stress can trigger oil accumulation in growth-inhibited *Chlamydomonas reinhardtii* algae (Siaut et al. 2011), in case of oil-seed plants its action is typically manifested by a dramatic loss of oil productivity (Francois 1994).

In contrast, BRs are known to enhance seed oil accumulation. For instance, end-point increase in oil content induced by tissue-specific photosynthesis in silique walls can be mimicked by application of BRs (Hua et al. 2012).

In our studies, application of 24-EBR during germination and vegetative growth did not sizeably affected seed productivity or oil content of seeds obtained from control *B. napus* plants (Fig. 2). However, 24-EBR application restored the seed yield of salt-stressed plants to 60 % of that of control plants. Besides, seed oil content in such conditions was matching that of normally-grown plants (Fig. 2).

This indicates a role for 24-EBR in modulating plant metabolic reactions, thus leading to alleviation of stress influence and increased plant productivity. Two explanations of the observed effect of 24-EBR should be considered. One possibility is that 24-EBR optimises plant growth and increase photosynthesis efficiency during stress. Another possibility is that exogenous BR treatment might subdue some of the plant basal activities or developmental programmes for sake of increased productivity. However, germination rate of seeds obtained from BR-treated plants was unaffected (data not shown). Being lipophilic compounds, BRs may also be targeted to membranes where they contribute to stress-induced adaptation.

Influence of salt stress and 24-EBR treatments on fatty acid composition of seed oil in *B. napus* and *A. thaliana* model plant

The FA composition of seed oil is a decisive factor of oil quality and usability both for biodiesel production and food purposes. We have analysed FA composition of seed oil from *Arabidopsis* model plants and *B. napus* crop plants belonging to the same *Brassicaceae* family (Fig. 3). FA compositions of seed oil obtained from both plant species were not significantly affected by 24-EBR treatment when

grown in auspicious conditions. In turn, in the salt-stressed *Arabidopsis*, an increase in 16:0 and 18:1 FAs content was accompanied by the decrease in other FAs abundance (Fig. 3). In *B. napus* changes of FA composition induced by salt stress were less pronounced with the slight decrease of 18:1 and 16:1 FAs content notable (Fig. 3). Several plant hormones are known to affect lipid FA composition. Auxins increased FA polyunsaturation ratio in phosphatidylcholine, triglycerides and free fatty acids, but not in glycolipids in cotyledons of soybean embryo (Liu et al. 1995). Gibberellins had influence on the level of saturated FA in phospholipids of heat-shocked aleurone layers of barley (Grindstaff et al. 1996). In contrast, 24-EBR was previously reported to not influence FA composition in plant seeds (Janeczko et al. 2009). However, in another study, BRs affected FA profile of polar lipids in mango fruits resulting in higher unsaturation degree beneficial in cold stress conditions (Li et al. 2012). We observed that 24-EBR application to plants subjected to salt stress have affected content of several FAs (Fig. 4). Hence, in the salt-stressed *B. napus* 24-EBR treatment induced over-accumulation of 18:2 FA. In the salt-stressed *Arabidopsis* application of 24-EBR resulted in partial reversion of 18:1 and 20:1 FAs content to that of a control plants. This may possibly indicate weakening of stress impact in 24-EBR-treated plants, as also suggested by increased productivity (Fig. 1).

It is known that the relationship between fatty acid biosynthesis and stress responses is reciprocal in plants. As an example, FA desaturase was reported to be involved in salt tolerance in *Arabidopsis* (Zhang et al. 2012) and changes in FA composition were demonstrated to be critical for maintaining membrane properties and fluidity in

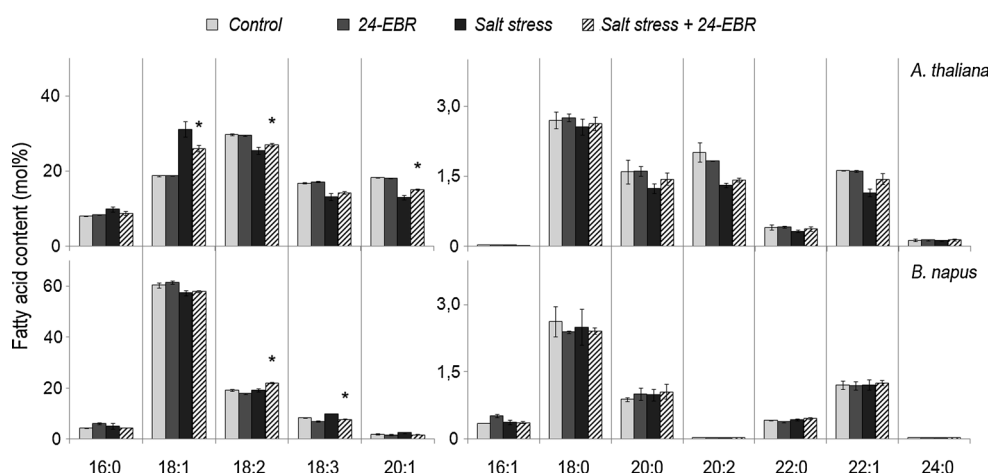


Fig. 3 Changes in fatty acid composition of seed oil from *A. thaliana* and *B. napus* subjected to different cultivation conditions. Oil samples were extracted from seeds gathered at the end of vegetative period and analysed by HPLC. Plant treatment conditions were identical to those described previously. A statistical comparison (Student's *t* test)

was performed for data obtained from salt-stressed plants and salt-stressed plants treated by 24-EBR. Data represent mean \pm SE, $n = 3$; *, $P < 0.05$, Student's *t* test, $n = 3$ discrete samples from two biological experiments

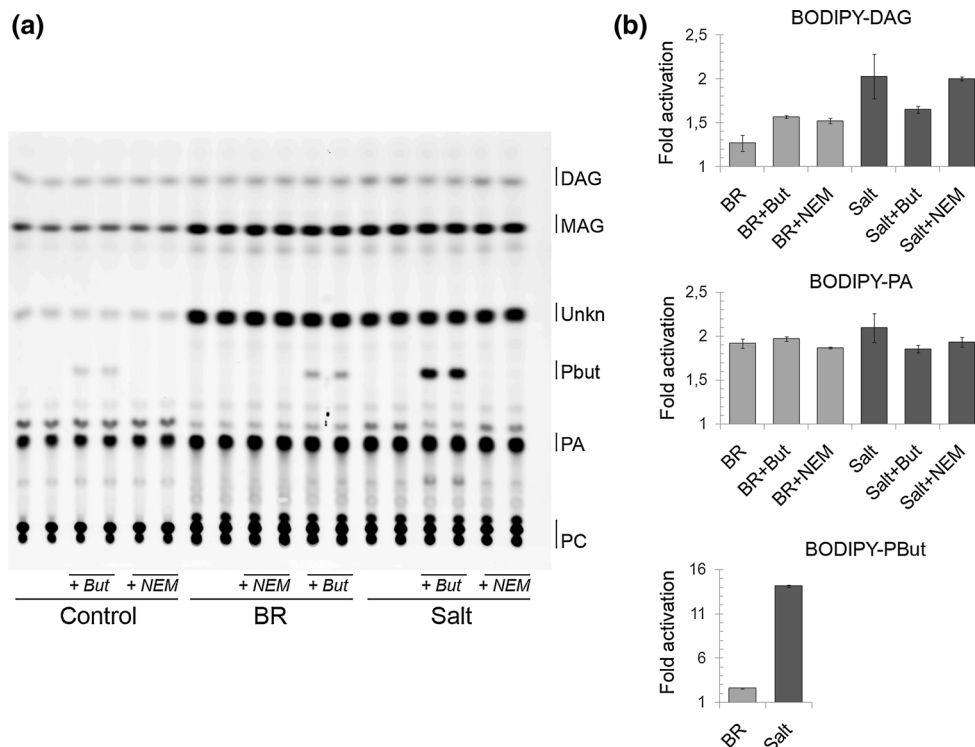


Fig. 4 Activation of phospholipid signalling in *B. napus* seedlings subjected to salt stress and 24-EBR. *B. napus* seedlings were hydroponically grown for 7 day. Seedlings were labelled with BODIPY-PC for 10 min and then 1 μ M 24-EBR (BR) or 100 mM NaCl (Salt) were added as indicated. Control plants were treated with buffer. *N*-ethylmaleimide (NEM) and *n*-butanol (But) were added to the incubation buffer prior to stress treatment. After 2 h lipids were extracted and separated by TLC. **a** Chromatography plate.

b Quantification of fluorescence associated with BODIPY-DAG, BODIPY-PA and BODIPY-PBut spots. The fluorescence was measured and expressed as fold change of that in control samples. Data represent mean \pm SE from independently analysed parallel samples $n = 2$. DAG, diacylglycerol; MAG, monoacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; Pbut, phosphatidylbutanol; Unkn, unknown product

stress conditions (Román et al. 2012). That is why the role of BRs in plant stress responses may be associated with adaptive changes of lipid FAs. Nevertheless, it should be noted that fatty acid composition of storage TAGs is usually distinct from that of membrane lipids.

Lipid signalling is activated by 24-EBR and upon salt stress in *B. napus*

Both abiotic and biotic stresses are known to evoke complex responses and defence reactions in plants (Atkinson and Urwin 2012). Here we investigated whether 24-EBR and stress conditions stimulate lipid signalling pathways in *B. napus*. Roots of 7-day-old plantlets were labelled with fluorescently labelled BODIPY-phosphatidylcholine for 10 min (Pejchar et al. 2010). Then 100 mM NaCl or 1 μ M 24-EBR were added in cultivation media for 2 h before extracting and separating lipids by thin layer chromatography (Fig. 4). Both treatments led to a substantial increase in fluorescent DAG and PA accumulation. This may suggest the activation of plant non-specific phospholipase C and/or phospholipase D enzymes that catalyse the hydrolysis of

phosphatidylcholine (PC) into DAG and PA, respectively (Hong et al. 2008; Kocourková et al. 2011; Krinke et al. 2009). In our experiments we have also studied the effects of two substances that affect lipid signalling. *N*-ethylmaleimide (NEM) is a thiol-reactive inhibitor of phosphatidate phosphatases that perform PA dephosphorylation to DAG (Furukawa-Stoffer et al. 1998). In turn, *n*-butanol (But) is used by PLDs as a substrate in the so-called transphosphatidyl reaction (Rainteau et al. 2012) thus leading to the production of phosphatidylbutanol (PBut) detrimentally to PA production. PBut production is thus considered as a marker of PLD activity (Kravets et al. 2010). In our investigations, we have observed limited or no effects of NEM on DAG and PA accumulation (Fig. 4). This indicates that DAG is likely to arise via a direct action of NPC on PC rather than via the dephosphorylation of PA. Treatment with *n*-butanol invoked slight decrease in PA production in salt stressed plants but not in plants treated with 24-EBR (Fig. 4). More importantly, these changes were markedly accompanied by the significant accumulation of PBut. To our knowledge, this is the first report demonstrating PLD activation in response to brassinosteroid treatment. Whether a post-translational

activation of PLD or a regulation of PLD gene expression both contribute to the increased PLD activity in response to BRs is a question of further studies. It is also remain to be investigated whether BR treatment affects activity of diacylglycerol kinases that were identified as major contributors to PA production in plants (Arisz et al. 2013).

Lipid signalling is often regarded to be critical for plant stress-tolerance as was demonstrated for salt-sensitive PLD-deficient *Arabidopsis* mutants (Hong et al. 2008). Our results indicate that both BR and salt stress activate unidirectional lipid signalling. The way plant cell distinguish related signals evoked by dissimilar stimuli is not well understood. It is thought that spatial and temporal parameters of the second-messenger production by different phospholipases isozymes are important. Different composition of fatty acid residues in such signalling molecules may also stand for dissimilarity in evoked cellular responses (Maatta et al. 2012; Rainteau et al. 2012).

Plant lipid metabolism and oil accumulation is linked with BR/phospholipid signalling and plant stress responses

Here we provide an *in silico* analysis pointing at integration of plant lipid metabolism with BR/phospholipid signalling and stress responses at transcriptional level using Genevestigator interface (Hruz et al. 2008). Rapeseed genome has been reported to be sequenced by Bayer Crop science. However, the size and complexity of allotetraploid *B. Napus* genome and the lack of available array data make it challenging to conduct efficient *in silico* studies for this species. Instead, we have analysed gene expression data of the taxonomically related model plant *A. thaliana* (Fig. 5). Numerous genes that are involved in the lipid metabolism have their expression modified in response to BR and salt stress. As an example, *KCS1* and *FAR1*, genes encoding proteins implicated in FA biosynthesis, were identified to be BR-responsive (Goda et al. 2002). Interestingly, the expression of *3-KETOACYL-COA SYNTHASE 9 (KCS9)*, *GLYCOSYLPHOSPHATIDYLINOSITOL-ANCHORED LIPID PROTEIN TRANSFER GENE 2 (LTPG2)* and GDSL-LIKE LIPASE genes were repressed in salt stressed plants but activated after BR treatment. *FATTY ACID REDUCTASE 1 (FAR1)* gene demonstrated reverse regulatory pattern. In addition two key genes of fatty acid metabolism - *FATA ACYL-ACP THIOESTERASE (FATA1)* and *FATTY ACID ELONGATION1 (FAE1)* were affected by BR and salt stress unidirectional. These available data provide hints at putative targets of BR for controlling the lipid productivity in stress conditions.

BRs also affect the expression of several genes that are directly implicated in plant stress responses. Among them are *DREB2A*, *RD29B* and *RAB18*. This may suggest that BRs can affect plant lipid productivity indirectly by boosting

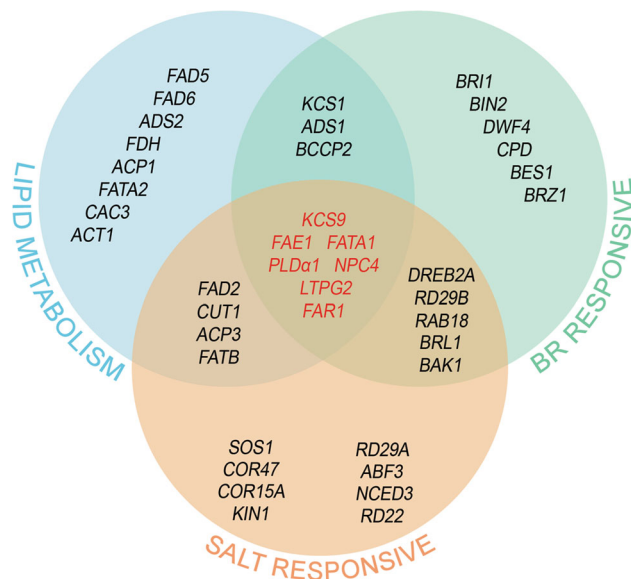


Fig. 5 Genetic association of lipid metabolism with BR-signalling and salt stress responses. Given here is a diagram representing *A. thaliana* genes implicated to lipid metabolism and signalling that show responsiveness to salinity conditions or treatment with brassinosteroids (as shown in circle intersections). Genes that potentially facilitate BR-mediated regulation of lipid metabolism in salt stress conditions are marked in red (Hruz et al. 2008). Common gene names are shown (for details see Electronic supplementary material Table 1). Experimental data used (GEO numbers): salt treatment AT-00120, brassinosteroid treatment AT-00110. Representative lipid metabolism genes were selected from (Beisson et al. 2003)

overall plant viability during stress (Clouse 2011). It was recently reported that expression of *DREB2A* is negatively regulated by the basal activities of phospholipase D and phospholipase C coupled to DGK (Djafi et al. 2013). This is an indication of the links between *DREB2A* genetic pathway and lipid signalling. This opens the possibility that BRs affect *DREB2A* expression through altering lipid signalling. In support, expression of several phospholipase genes (namely *PLDα1* and *NPC4*) is also responsive to BRs and salt stress stimuli, which suggests their implication in the control of plant stress responses. Biochemical cues are also indicating that NPC activity is sensitive to BRs application in plants (Wimalasekera et al. 2010). It was also suggested that BRs act in concert with phospholipase A₂-derived lysophosphatidylethanolamine phospholipid to regulate growth of primary roots and the formation of lateral roots in *Arabidopsis* (yoon Jeong et al. 2012).

The integration of phospholipases to lipid metabolism is now well ascertained. PA derived presumably from PC, as a major constituent of plant membranes, is important for lipid turnover and tobacco pollen tube growth (Pleskot et al. 2012). In turn, DAG molecules produced by NPC are common precursors to both membrane PC and storage TAGs (Pokotylo et al. 2013). Interestingly, phospholipases are abundant in seeds. PLD was shown to be active

throughout most parts of seed development while germination and storage TAGs composition was significantly affected in PLD α -deficient mutants of soybean (Lee et al. 2012). It is becoming apparent that not only phospholipases participate in immediate stress signalling but actively contribute to lipid turnover in plant leaves and, more importantly, seeds.

Conclusions

In summary, our results suggest that treatment of *B. napus* plants with BRs leads to the increased seed oil productivity in stress conditions. We have also observed that BRs and salt stress activate phospholipid signalling. This data suggest that phospholipases are among the cell components that mediate BRs signal. Gene expression analysis in publicly available database also provided a hint at an association of lipid metabolic processes with BRs and phospholipases. The question how BR signal is mediated downstream of lipid signalling implicating PLD and NPC phospholipases in plant cells is currently unanswered. Though some clues can be provided by the identification of DAG-sensitive or PA-sensitive intracellular targets (McLoughlin et al. 2013).

Not only our results provide evidences for BRs role in the control of lipid signalling and lipid metabolism, but they grant basis for further research required for steady development of biofuel production.

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