ORIGINAL ARTICLE

Triterpenoid modulates the salt tolerance of lanosterol synthase deficient *Saccharomyces cerevisiae*, GIL77

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Abstract This study examined the effect of triterpenoid on the salt tolerance of lanosterol synthase deficient yeast mutant GIL77. The expression of the triterpenoid synthase gene under *GAL1* promoter in GIL77 increased the triterpenoid concentration of both whole cell and plasma membrane fractions. Without the induction of the genes, the growth curve of *BgbAS* or *RsM1* transformant depicted patterns similar to control cells in both the presence and absence of salt with growth inhibition at 500 mM NaCl. The induction of *BgbAS* and *RsM1* gene expression slightly repressed growth compared with control cells in the absence of NaCl. The growth of GIL77 was significantly suppressed by the expression of *BgbAS* or *RsM1* under salinity conditions. Of the triterpenoid synthase genes, *BgbAS* rather than *RsM1* was found to strongly inhibit the growth of GIL77 cells under salt stressed conditions. The expression of the triterpenoid synthase gene in GIL77 also influenced their tolerance to other abiotic stresses. In contrast to the endogenous synthesis, the exogenous supply of triterpenoid in the culture medium appeared to occur in the plasma membrane fraction and enhanced the salt tolerance of GIL77. This study thus discussed the physiological significance of triterpenoid in relation to its possible role in modulating salt tolerance.

1. Introduction

Mangrove plants are unique in that they can grow under a wide range of salinity conditions, ranging from freshwater to a hypersaline environment (Tomlinson, 1986). Several types of salt tolerance mechanisms have been proposed for mangrove plants: (1) adjustment of osmotic pressure by the accumulation of small molecule osmolytes, such as glycinebetaine or sugar alcohol (Popp, 1984; Sakamoto and Murata, 2000); (2) salt extrusion across the plasma membrane using an ion transporter (Allen et al., 1995); (3) compartmentalization of salt in the vacuole

(Blumwald and Poole, 1987; Mimura et al., 2003); and (4) expression of certain functional genes to neutralize the salt toxicity (Sugihara et al., 2000; Yamada et al., 2002). In addition to these well-documented explanations, we observed increases in triterpenoid concentration and triterpenoid synthase gene expression in mangrove plants with increasing salt concentration (Basyuni et al., 2009; Oku et al., 2003). Surveys of salt tolerance gene expression by our and other studies suggested that triterpenoids are involved in the late stage of the salt tolerance mechanism in mangrove plants, being integrated as a component of long-term adaptation in combination with other short-term regulation mechanisms to confer salinity tolerance (Basyuni et al., 2012a; Ezawa and Tada, 2009; Yamanaka et al., 2009). This salt-dependent change in triterpenoid concentration was reversible upon transfer of the plants to fresh water (Basyuni et al., 2012b). Furthermore, our previous study found that the basal level of triterpenoid concentration in mangrove species correlated well with their habitat zonation along the coast to the inner-island axis: mangrove species growing closer to the sea showed higher triterpenoid concentrations (Basyuni et al., 2012a).

Fig. 1 depicts the biosynthetic pathway of triterpenoids and sterols. Triterpenoids and sterols are biosynthesized from a common precursor (2,3-oxidosqualene) by the enzyme oxidosqualene cyclase (OSC). Phytosterol, but not triterpenoid, has been accepted as a sterol component of plasma membrane in the plant kingdom. Several studies have demonstrated the biological activities of triterpenoid and their derivatives (Liu, 2005; Montilla et al., 2003; Safayhi and Sailer, 1997), and these biological activities have been explained by the disruption of membrane integrity caused by insertion of foreign molecules such as triterpenic acid into membrane phospholipid bilayers

(Prades et al., 2011). It is therefore possible that the plasma membrane can accommodate a wide array of lipid molecules to some extent, depending on their physicochemical properties (Nes et al., 1993). Thus, it appears plausible that triterpenoid could replace phytosterol in the plasma membrane of mangrove plants.

On the basis of the series of our and other studies, we hypothesized that triterpenoids incorporate into the plasma lipid membrane, thereby changing salt stress tolerance. The scenario we proposed as mentioned above requires further testing, particularly in terms of physiological functions of terpenoids in the salinity stress using a model microorganism or plant. To introduce the triterpenoid synthase gene and analyze the function of their OSCs, lanosterol synthase (LS) deficient *Saccharomyces cerevisiae* GIL77 was used as the host organism (Basyuni et al., 2006; Kushiro et al., 1998). This host organism cannot synthesize ergosterol, a component of their cell membranes in yeast, and accumulates 2,3-oxidosqualene inside cells (Fig. 1). Therefore, transformation of GIL77 with other OSC genes and its expression results in the conversion of the substrate 2,3-oxidosqualene to the corresponding reaction products, depending on the activities of the OSCs. Thus, the present study investigated the effect of triterpenoid in GIL77 on its tolerance to abiotic stress, including salinity.

2. Materials and methods

2.1. Chemicals

β-amyrin and β-sitosterol were purchased from Extrasynthese (Rhône, France). All lipids were dissolved in hexane, and stored at −30 °C until use.

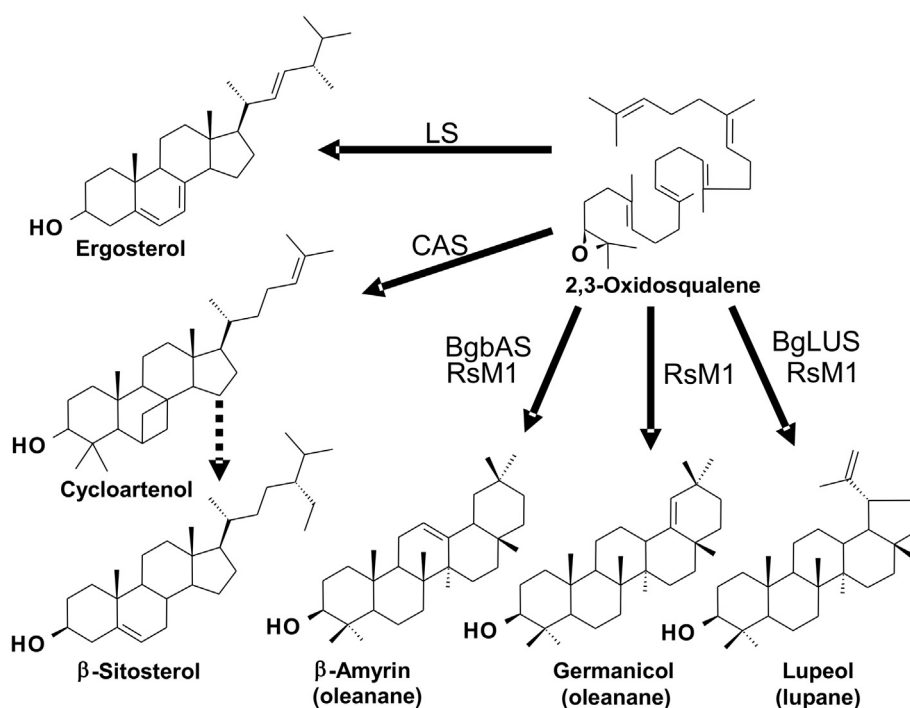**Figure 1** Cyclization of 2,3-oxidosqualene to terpenoids and phytosterol in mangrove trees. Terpenoids and phytosterol are biosynthesized from a common precursor by the enzyme oxidosqualene cyclases. LS, lanosterol synthase; CAS, cycloartenol synthase; *BgbAS*, *Bruguiera gymnorrhiza* β-amyrin synthase; *BgLUS*, *Bruguiera gymnorrhiza* lupeol synthase; *RsM1*, *Rhizophora stylosa* multifunctional triterpene synthase.

2.2. Yeast strain and culture condition

Saccharomyces cerevisiae strain GIL77 (*gal2 hem3-6 erg7 ura3-167*) lacking LS activity was used as the host organism to introduce the triterpenoid synthase genes. For the culture of *S. cerevisiae*, all synthetic complete (SC) media used in the present study contained 13 μg/mL hemin, 20 μg/mL ergosterol, and 5 mg/mL Tween 80. Our previous studies cloned triterpenoid(s) synthase genes from mangrove plants; the composition of their reaction products are listed in Table 1 [data were taken from the Table of reference (Basyuni et al., 2007)]. *BgLUS* and *BgbAS* were monofunctional and respectively produced single triterpenoids of lupeol and β-amyrin. In contrast, *RsM1* was multifunctional and produced β-amyrin, germanicol, and lupeol at a molar ratio of 33/63/4. Introduction of *KcCAS*, cycloartenol synthase, into GIL77 resulted in a very low level of reaction products; hence, its effect on salt tolerance was not a focus of the present study (data not shown). These genes were ligated into the high copy number-shuttle vector, pYES2 (Life Technologies Japan Ltd., Tokyo, Japan) under the control of *GAL1* promoter. The plasmid was transfected into GIL77 using the Frozen-EZ yeast transformation II kit purchased from Zymo Research (CA, USA). The empty pYES2 vector was also introduced into GIL77 as a control.

2.3. Stress tolerance assays with transformants

GIL77 transformed with a vector harboring triterpenoid synthase gene or empty vector was pre-cultured overnight at 30 °C in SC-Ura liquid medium containing 2% glucose (SCGlc-Ura medium). To induce the OSC gene driven by *GAL1* promoter, the transformant was inoculated at 0.05 of OD_{600} into 1 mL of SC-Ura containing 2% galactose (SCGal-Ura medium), and cultured for 24 h at 30 °C. After induction of OSC gene expression, equal numbers of cells were cultured in the presence or absence of sodium salt (250 or 500 mM), and the growth was monitored by OD_{600}. In the case of the reference experiment without induction of the triterpenoid synthase gene expression, SCGal-Ura was replaced with SCGlc-Ura medium.

To test the elevated level of triterpenoid on the tolerance to other abiotic stresses such as metal ions and osmolytes, transformants were cultured at 30 °C for 24 h in SCGal-Ura medium with supplementation of 0.5 M NaCl, 1.5 M KCl, 0.5 M $MgCl_2$, 2.5 mM $CuCl_2$, 2.5 mM $CuSO_4$, or 2.3 M sorbitol. In the case of oxidation stress, transformant was incubated with 0.1 mM H_2O_2 for 1 h. For heat stress, the cultures were maintained at 45 °C for 3 h. Cell number after these treatments was estimated by their growth on SCGlu-Ura agarose plate after serial dilution.

2.4. Growth of GIL77 under exogenous supply of terpenoids

β-sitosterol or β-amyrin were added to SC containing galactose (SCGal) at a final concentration of 20 μg/mL, and were solubilized by autoclaving. To test the salt stress tolerance, GIL77 (non-transformants) was inoculated at 0.001 of OD_{600} into the above terpenoid supplied SCGal containing 500 mM of NaCl, and the growth at 30 °C was monitored.

2.5. Isolation of plasma membranes

Plasma membrane was purified from yeast cells by the previously reported method (Abe et al., 2009) with slight modifications. The GIL77 (1×10^9 cells) collected at the logarithmic growth phase were washed with TE buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA) twice, and broken in tris-ethylenediaminetetraacetic acid (TE) buffer with glass beads. The lysate was combined with an equal volume of 2-morpholinoethanesulfonic acid (MES) buffer (50 mM MES, 1 mM $MgCl_2$, pH 5.2). The mixture was centrifuged at 1000g for 5 min and 3000g for 5 min to remove the cell debris and mitochondria respectively, and at 13,000g for 10 min to obtain the membrane pellet. The resulting supernatant was collected as intracellular fluid. The pellet was washed twice in TE/MES buffer (1/1, by vol.) and suspended in 1 mL TE buffer. To isolate the plasma membrane, the suspension was gently placed on top of 20%, 40%, and 50% stepwise sucrose gradient, and centrifuged at 100,000g for 18 h at 4 °C on a himac CP85β using a P40ST rotor (Hitachi Koki Co., Ltd., Tokyo, Japan). The resultant plasma membrane pellet was stored at −30 °C until use.

2.6. Lipid extraction, thin layer chromatography (TLC), and gas chromatography (GC)

The cell pellet, intracellular fluid, and plasma membrane pellet were saponified by reflux with 0.1 M KOH in 50% ethanol at 90 °C for 10 min. Non-saponifiable lipids in the lysate were extracted with the same volume of hexane and concentrated. To analyze the lipids produced by the action of triterpenoid synthases, the concentrated extracts were applied onto a silica gel TLC plate (Merck KGaA, Darmstadt, Germany), followed by development with benzene/acetone (19/1, v/v). To visualize the chromatograms, the TLC plates were immersed in phosphoric acid/33% acetic acid/sulfuric acid/0.5% copper sulfate (5/5/0.5/90, by vol.) for 10 s, and heated at 140 °C for 15 min. The band intensities were analyzed by densitometry using the ImageJ software (US National Institutes of Health, http://rsb.info.nih.gov/ij/download.html). To analyze the exogenously supplied lipids in the plasma membrane of GIL77 non-transformants, the samples were analyzed using GC with a flame ionization detector (FID) (GC-2010, Shimadzu Corp., Kyoto, Japan). The column used was CBP1-M50-025 (0.25 mm ID × 50 m, Shimadzu) with the temperature program set at 50 °C for 1 min, then raised to 300 °C at a rate of 10 °C/min, and maintained at 300 °C for 26 min. The carrier gas was He with a flow rate of 20 cm/s, and the temperature for the injector and detector were 250 °C and 300 °C, respectively.

Total cellular lipids were extracted and purified by the previously reported method (Bligh and Dyer, 1959). For the

Table 1 The product profiles of triterpene synthases used in this study.

Component	*Bruguiera gymnorrhiza*		*Rhizophora stylosa*
	BgbAS[†]	*BgLUS*[†]	*RsM1*[†]
β-amyrin	100%	–	33%
Germanicol	–	–	63%
Lupeol	–	100%	4%

[†] *BgbAS*, β-amyrin synthase; *BgLUS*, lupeol synthase; *RsM1*, multifunctional triterpenoid synthase.

separation of phospholipids, 50 .g of total lipids were sepa-
rated on a silica gel TLC plate (10 × 10 cm) as described pre-
viously (Ponec et al., 1988) with slight modification. Briefly, we
used chloroform/acetone/methanol (90/5/5, by vol., up to
4 cm) as the first developing solvent. A solution of chloro-
form/ethyl acetate/methanol/2-propanol/triethylamine/water
(64/14/17/0.5/3/2, by vol.) was used as the second and third
developing solvent up to 9 and 6 cm, respectively. The lipids
were visualized and analyzed by the methods described above.

2.7. Statistical analyses

All values are expressed as the mean ± standard error of the
mean (S.E.M.). The significances of the differences among
means for more than three groups were inspected using Dun-
nett's test. Differences were considered to be statistically signif-
icant at $P < 0.05$.

3. Results

Fig. 2 shows the TLC chromatograms of non-saponifiable
lipids extracted from GIL77 transformed with *BgLUS*,
RsM1, or *BgbAS*. Expression of the triterpenoid synthase gene
increased the intracellular levels of triterpenoid. However, the
extent of increase showed variation depending on the genes, to
a greater extent for *RsM1* and *BgbAS* and to a lesser extent for
BgLUS. The relative band intensity of triterpenoid against
ergosterol on TLC was 0.1, 0.9, and 0.8 for the reaction prod-
ucts of *BgLUS*, *RsM1*, and *BgbAS*, respectively.

With no gene induction, the growth curve of *BgbAS* or
RsM1 transformant depicted a similar pattern as the control
cells in both the presence and absence of salt, whereas the
growth was equally inhibited by 500 mM NaCl (Fig. 3A).
Meanwhile, induction of *BgbAS* and *RsM1* gene expression
via the activation of *GAL1* promoter significantly affected
the growth of GIL77 (Fig. 3B). Statistically significant but
slight growth delays of *BgbAS* and *RsM1* transformants were
observed when compared with control cells at the 48 h culture,
even in the absence of NaCl (Fig. 3B, upper panel). The
growth of GIL77 was significantly affected by the expression
of *BgbAS* or *RsM1* under the salinity stress conditions
(Fig. 3B, middle and lower panel). Significant growth delays
of *BgbAS* and *RsM1* transformants were observed when cul-
turing in the presence of 250 mM NaCl after 36 h of culture.
Of the triterpenoid synthase genes, *BgbAS* rather than *RsM1*
was found to show much stronger inhibition of the growth
of GIL77 cells. In comparison with control cells, the growth
delay of *RsM1* transformant was observed in the presence of
500 mM NaCl, just as was the case of 250 mM NaCl. The
growth curve patterns of *BgbAS* transformant were distinctly
different between the salinity conditions at 250 and 500 mM.
Although the control cells and *RsM1* transformant appeared
to enter a stationary phase at approximately 60 h of culture,
BgbAS transformant grew slowly throughout the experimental
period.

Fig. 4 shows the effect of terpenoid synthase gene expres-
sion on the tolerance of GIL77 to other abiotic stresses such
as toxic metal ions, osmolyte (sorbitol), oxidant (H_2O_2), and
heat. The growth of transformant with triterpenoid synthase
genes *BgLUS*, *BgbAS*, or *RsM1*, were largely comparable to
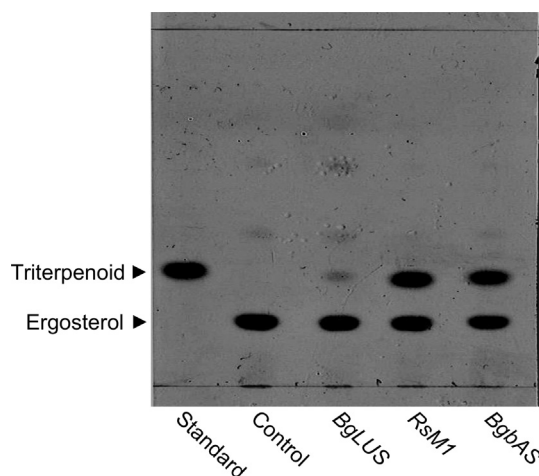that of control cells in the presence of sorbitol and $MgCl_2$.

Figure 2 Triterpenoid production in GIL77 yeast cells overex-
pressing oxidosqualene cyclases (OSC) genes. GIL77 yeasts
transformed with a vector harboring *BgLUS*, *RsM1*, *BgbAS*, or
an empty vector (control) were incubated in SCGal-Ura medium
for 24 h at 30 °C. Lupeol was used as a standard of triterpenoid.

Consistent with the result of our forgoing experiment
(Fig. 3), the expression of *BgLUS*, *BgbAS*, and *RsM1* lowered
the growth of GIL77 in the presence of sodium salt, with
greater inhibition by *BgbAS* compared with the other genes.
Largely the same extent of growth inhibition by the expression
of *BgbAS* was noted for toxic metal ions of copper ($CuCl_2$ and
$CuSO_4$). Three triterpenoid synthase genes equally decreased
the resistance of GIL77 against the oxidation stress of 1 mM
H_2O_2. In contrast to the aforementioned growth inhibition
by the expressions of OSCs, it was likely that expression of
triterpenoid synthase genes protected the cells from heat stress.
The expression of *RsM1*, but not the other genes, inhibited the
cell growth in the presence of 1 M KCl compared with the
control.

To ascertain the incorporation of triterpenoid into mem-
brane lipid, its intracellular distribution in GIL77 was studied
(Fig. 5A). Transformation of GIL77 with triterpenoid syn-
thase gene increased the concentrations of triterpenoids in
intracellular fluid and the plasma membrane, as well as the
entire cell body. The levels of triterpenoids in the whole cell
body were largely similar to the result shown in Fig. 2. The
concentration of triterpenoid in the intracellular area of the
plasma membrane was lower than that of the whole cell body.
Analysis of band intensities on TLC found that ratios of triter-
penoid concentration to ergosterol in plasma membrane were
0, 0.18, and 0.11 for the transformant of *BgLUS*, *RsM1*, and
BgbAS, respectively. The expression of *BgLUS* resulted in
rather lower accumulation of triterpenoids in both intracellu-
lar fluid and plasma membrane compared with the cases for
BgbAS and *RsM1*, primarily due to the lower gene expression
or catalytic efficacy of the enzyme protein to convert the sub-
strate to lupeol. The relative concentrations of glycerol-
phospholipids were largely comparable between control and
BgbAS transformants (Fig. 5B).

To examine the effects of exogenous supply of triterpenoid
on salt tolerance, GIL77 cells were grown in the medium sup-
plemented with β-sitosterol or β-amyrin (Fig. 6A). The occur-
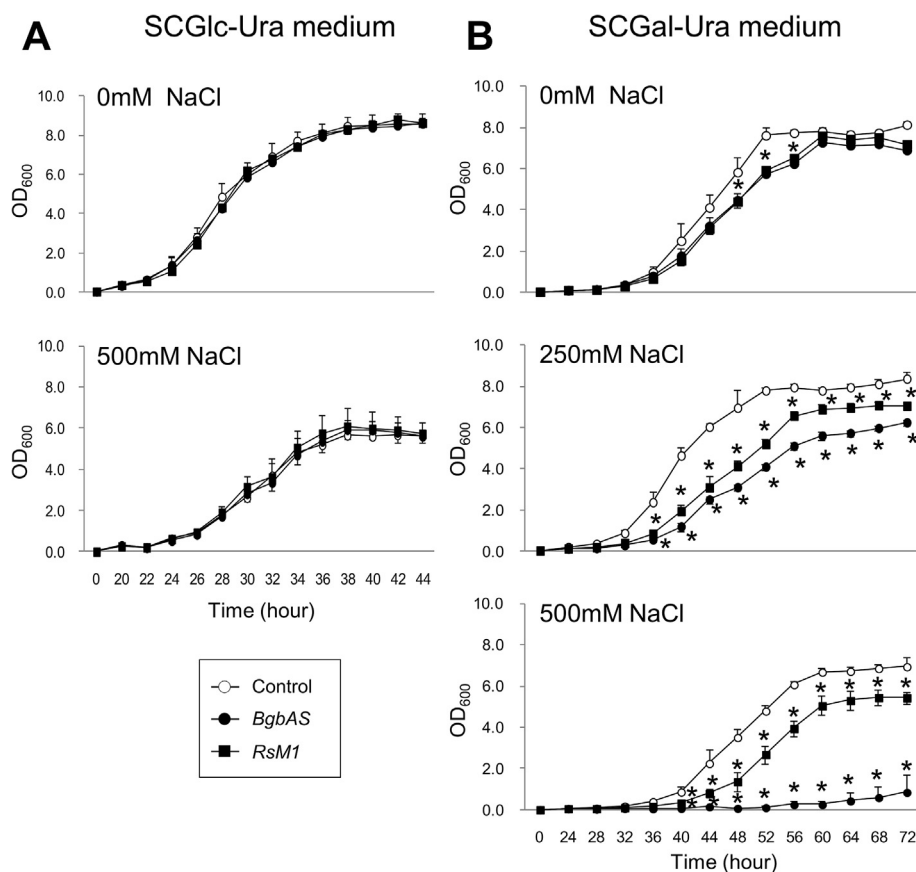rence of β-amyrin slightly decreased the growth rate of GIL77

Figure 3 Growth of GIL77 transformants in the presence or absence of salt. GIL77 yeasts transformed with a vector harboring *RsM1*, *BgbAS*, or an empty vector (control) were incubated at 30 °C in SCGlc-Ura (A) and SCGal-Ura (B) media. The data are expressed as the mean ± standard error of the mean (S.E.M.) for three clones.

in the absence of NaCl, whereas inclusion of β-sitosterol had little effect on the growth. However, the situation completely reversed in the presence of salt stress. β-amyrin enhanced the cell propagation in the presence of 0.5 M NaCl, whereas β-sitosterol decreased the growth as compared with the control. GC-FID analyses determined whether exogenously supplied lipid exists in the plasma membrane of GIL77 non-transformant (Fig. 6B). The ratios of exogenously supplied lipid concentration to ergosterol in the plasma membrane were 0.19 and 0.20 for GIL77 grown in the presence of β-sitosterol and β-amyrin, respectively.

4. Discussion

The present paper studied the effect of triterpenoid on the salt tolerance of LS deficient mutant *S. cerevisiae* GIL77. Gene expression of triterpenoid synthase in GIL77 elevated the intracellular and plasma membrane levels of triterpenoid (Figs. 2 and 5A). An exogenous supply of triterpenoid or phytosterol also elevated their own plasma membrane levels (Fig. 6B). The increase in cellular triterpenoid concentration weakened their tolerance to salt stress (Fig. 3) and also affected tolerance to other physicochemical stresses such as oxidation or heat (Fig. 4). In contrast, the exogenous supply of triterpenoid enhanced the growth rate of GIL77 in the presence of salt stress (Fig. 6A). On the basis of our previous and cur-

rent data, in the present paper, we mainly discuss the novel physiological function of triterpenoid in salt tolerance, with emphasis on its interaction with plasma membrane as a potential membrane structure modulator.

There have been several notions that the plasma membrane is the first line of defense against changes in physicochemical variables of the thriving environment (Liu et al., 2013; Turk et al., 2011). It has also been suggested that the plasma membrane might be a primary site of salt injury (Mansour and Salama, 2004). Therefore, the integrity and functionality of the membrane is a critical factor for salt tolerance (Apse and Blumwald, 2002; Chen et al., 2001). In this context, the fluidity of the plasma membrane is an important determinant of salt tolerance, and can be used as an indicator of adaptation for survival in an extreme environment (Mansour, 2012). It has also been reported that a high level of NaCl increased the sterol to phospholipid ratio, but did not significantly impact the membrane fluidity in a marine yeast (Turk et al., 2007). In the case of yeast, the major sterol is ergosterol, and the abundance of this sterol in the plasma membrane is a coping factor for salinity stress (Hosono, 1992). In the present study, expression of triterpenoid synthase gene significantly increased the intracellular triterpenoid to almost the same level as ergosterol (Figs. 2 and 5A), but did not affect the composition of cellular phospholipids (Fig. 5B). The endogenous triterpenoid produced by the action of triterpenoid synthase and the exoge-
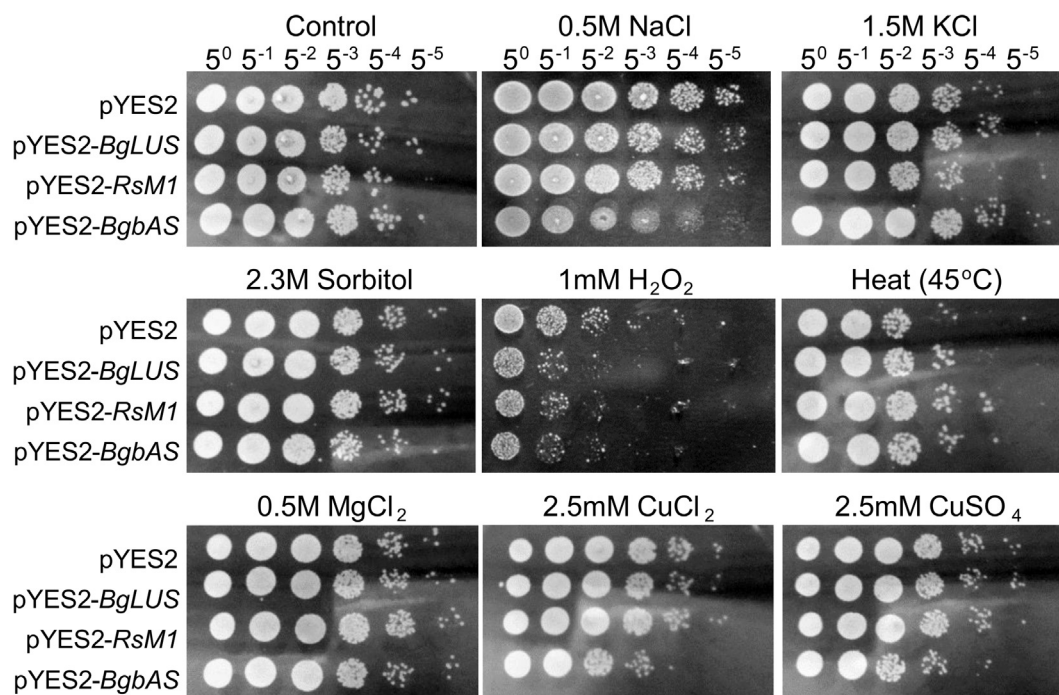
Figure 4   Growth of GIL77 transformants under abiotic stresses. GIL77 yeasts transformed with a vector harboring *BgLUS*, *RsM1*, *BgbAS*, or an empty vector (pYES2) were incubated in SCGal-Ura medium. An equal number of cells were treated in the appropriate media, and sequentially spotted on SCGlu-Ura agar-plate.
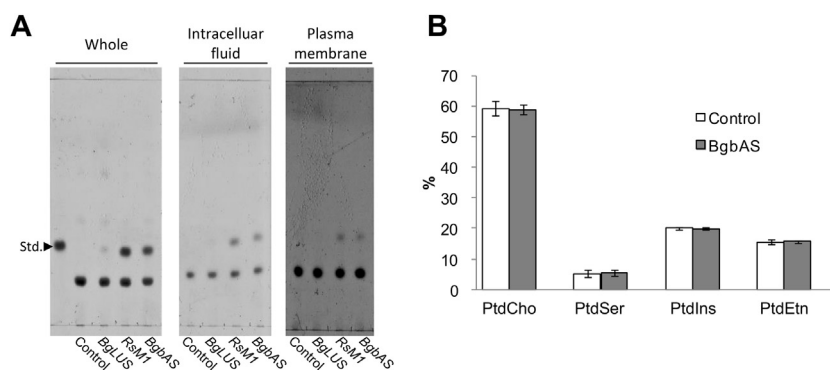

Figure 5   Triterpenoid and phospholipid composition of GIL77 transformants. Intracellular distribution of endogenous terpenoid (A) and phospholipid composition (B) of whole GIL77 transformant. GIL77 yeasts transformed with a vector harboring *BgLUS*, *RsM1*, *BgbAS*, or an empty vector (control) were incubated in SCGal-Ura medium. Std, terpenoid standard (lupeol); PtdCho, phosphatidyl-choline; PtdSer, phosphatidylserine; PtdIns, phosphatidylinositol; PtdEtn, phosphatidylethanolamine. The data are expressed as mean ± standard error of the mean (S.E.M.) for three clones.

nously supplied triterpenoid appeared to be located in the plasma membrane fraction, as shown in Figs. 5A and 6B. The X-ray diffraction study revealed that triterpenoic acid altered the structural properties of the phospholipid or phospholipid-cholesterol rich model membrane (Prades et al., 2011). The triterpenic acids were therefore likely to affect the lateral domain heterogeneity of the cell membrane. The physiochemical property of triterpenoids is somewhat different from that of ergosterol, and the mixing of foreign molecules with the ergosterol in the lipid bilayer may significantly change the membrane integrity, and hence, the susceptibility to salinity stress. As mentioned above, yeast under salinity stress

requires the presence of a high concentration of ergosterol in the plasma membrane for survival. An increased intracellular triterpenoid may therefore impede the translocation of ergosterol from the cellular compartment to the plasma membrane by competing in the trafficking process with ergosterol.

The plasma membrane consists of lipids and proteins, and these components are spatially arranged in an effective way for optimum functionality (Verstraeten et al., 2010). Changes in any of these organizations can therefore significantly perturb the primary membrane function. Furthermore, it is possible that perturbation in the primary membrane function consequently alters the activities of the membrane-embedded
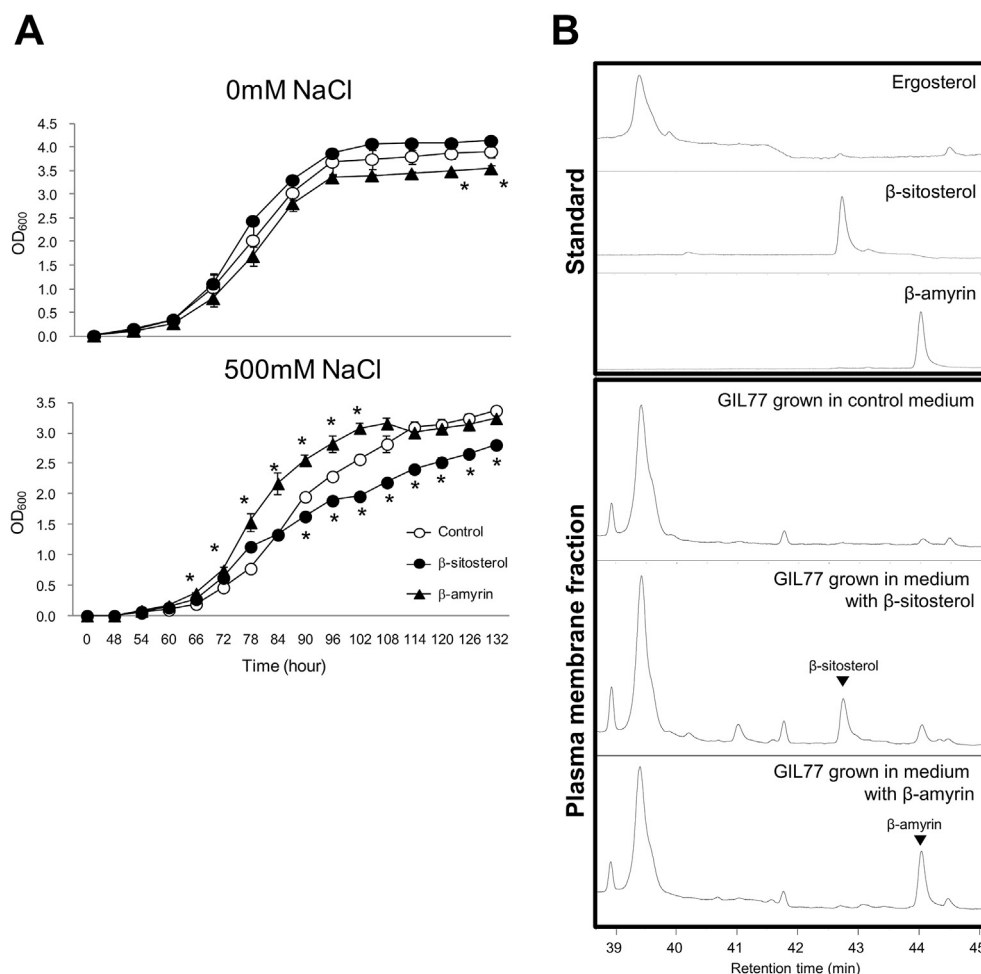
Figure 6 Effects of exogenous supply of triterpenoid or phytosterol on GIL77 non-transformants. Growth of GIL77 under salinity condition with exogenous supply of triterpenoid or phytosterol (A). The composition of non-saponifiable lipids in plasma membrane of cultured GIL77 (B). GIL77 non-transformant was cultured in SCGal medium supplemented with or without β-amyrin or β-sitosterol.

enzyme or proteins involved in the regulation of cell defense or permeability. A number of studies have demonstrated that the sterol is a modulator of permeability, enzyme activities, ion absorption, and fluidity of the membrane (Grunwald, 1968; Hartmann, 1998). In this context, it has been reported that plasma membrane H^+-ATPase activity influenced the tolerance of yeast against copper stress (Fernandes et al., 1998). One of the major small heat shock proteins residing in plasma membrane, Hsp12, is involved in the maintenance of membrane organization during several stress conditions (Sales et al., 2000; Varela et al., 1995). In the present study, the expression of the *BgbAS* gene significantly affected the growth of GIL77 under the copper, oxidative, and heat stresses (Fig. 4). Therefore, some uncertainties remain regarding whether changes to the composition and activities of stress tolerance related proteins in plasma membrane occur.

It is also worth noting that the molecular architecture of triterpenoid was an additional factor influencing the salt tolerance of GIL77. The presence of β-amyrin rather than germanicol or lupeol appeared to strongly inhibit salt tolerance in the present study (Figs. 3B and 4). Both β-amyrin and germanicol belong to the triterpenoid of oleanane type carbon skeleton,

and their chemical structures are very similar, as shown in Fig. 1. The difference between these two triterpenoids is the location of the double bond (Δ12 for β-amyrin, and Δ18 for germanicol). However, β-amyrin appeared to be more perturbative than germanicol, despite the subtle architectural difference between these two triterpenoids. In regard to free sterol, a notion exists that a greater amount of the planar type was involved in the membrane stabilization and reduction in membrane permeability (Grunwald, 1968; Mansour et al., 1994; Wu et al., 1998). The expression of triterpenoid synthase gene did not affect the cell growth in the presence of sorbitol (Fig. 4). This suggested that increased osmotic pressure did not necessarily explain the toxicity of NaCl in GIL77. Despite these results, further studies are required to clarify the effects of triterpenoids and its architecture on the salt tolerance in GIL77.

Endogenous supply of triterpenoid by the expression of the synthase gene impaired the salt tolerance of GIL77. In contrast, exogenous supply of triterpenoid by inclusion of this into the culture medium enhanced the salt tolerance. However, this is not the case for phytosterol (β-sitosterol). Supplementation of the medium with β-sitosterol significantly decreased the

growth rate in the presence of NaCl (Fig. 6A). The biphasic effect of triterpenoid on the salt tolerance, inhibition, and enhancement depending on the intra- or extra-cellular supply may be explained by the asymmetry of the plasma membrane. It has been established that the phospholipid composition of the inner and outer membrane leaflets differs, and an asymmetric phospholipid distribution can be generated and maintained across the plasma membrane (Quinn, 2002). It is likely that the insertion of triterpenoid from intracellular cytosol or extracellular culture medium modulated the physicochemical properties of the plasma membrane differently, so that their effects on salt tolerance contrasted.

In the present study as well as in previous studies (Prades et al., 2011), we mainly discuss the physiological significance of triterpenoid in salt tolerance, with emphasis on its interaction with plasma membrane as a potential membrane structure modulator. Previously, it has been reported that administration of triterpenoid or its ester ameliorated the cardiotoxicity of an antitumor or immunosuppressant agent in rats (Sudharsan et al., 2006). This cytotoxicity was ascribed to altered membrane permeability to electrolytes induced by the oxidative stress on cellular or membrane components. Triterpenoid has been proposed to normalize membrane permeability by intercepting the action of oxidant or free radicals and protecting the membrane phospholipid from peroxidation (Krishnaiah et al., 2011). However, the expression of triterpenoid synthase gene alleviated, but did not counteract, the oxidative damages caused by H_2O_2 and copper (Fig. 4). Furthermore, the biphasic mode of action of triterpenoid on the salt stress and their distinct activities depending on the subtle molecular architecture in the present study may refute its role as an anti-oxidant or radical scavenger to modulate salt tolerance of yeast. Further studies taking these two mechanisms into consideration are required to exploit the novel physiological function of triterpenoid. However, it is unlikely that triterpenoid modulated the salt tolerance of yeast via the prevention of plasma membrane from oxidation stress in the present study.

5. Conclusions

Herein, it is demonstrated, for the first time, that the endogenous or exogenous supply of triterpenoids affected the salt tolerance of LS deficient yeast, GIL77. We hope that this study triggers investigations into the physiological significance of triterpenoid in mechanisms of salt tolerance.

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