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Gibberellic acid (GA) role in acetyl-coA carboxylase enzyme regulation and in improving oil palm yield

Peran asam giberelat dalam regulasi enzim asetil-coA karboksilase dan peningkatan produksi minyak kelapa sawit

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Abstrak

Rumput laut, khususnya *Sargassum* sp., *mengandung hormon pemacu pertumbuhan yang telah terbukti meningkatkan pertumbuhan dan hasil tanaman karena kandungan auksin, asam giberelat (GA) dan sitokinin, serta beberapa asam amino khususnya asam glutamat. Kandungan tersebut dapat digunakan untuk menginduksi produksi minyak di kelapa sawit yang berhubungan dengan enzim asetil coA karboksilase, (ACC). Enzim ini merupakan langkah pertama dalam menentukan laju reaksi dalam jalur biosintesis asam lemak, menjadi aktif melalui defosforilasi beberapa residu serin yang diinduksi oleh magnesium dan glutamat. Selain itu, ACC diatur oleh interaksi AtWRI1 dan AtWRI1-TCP4, mekanisme yang memungkinkan penyesuaian jalur biosintesis minyak. Dalam penelitian ini dilakukan percobaan ekspresi gen dan analisis molekuler docking terhadap kemungkinan mekanisme komponen rumput laut yang dapat menstimulasi akumulasi minyak di kelapa sawit.Analisis lebih lanjut dilakukan untuk memastikan apakah interaksi antara TCP4 dan kandidat inhibitor mampu memfosforilasi TCP4 dan menurunkan aktivitasnya. Aplikasi GA menyebabkan peningkatan akumulasi minyak pada perlakuan 1 bulan, meskipun pada bulan kedua akumulasi minyak menunjukkan penurunan. Peningkatan akumulasi minyak pada bulan pertama sejalan dengan peningkatan ekspresi ACC pada minggu ke-3 dan ke-5. Sedangkan TCP4 menunjukkanpenurunanekspresiyang mengakibatkan peningkatan WRI1 pada minggu ke-5. Dari hasil ini dapat diindikasikan bahwa aplikasi GA mampu memblokir TCP4, sehingga TCP4 tidak dapat berinteraksi dengan WRI1 yang menyebabkan munculnya ekspresi WRI1 dan ACC. Interaksi ini merangsang akumulasi minyak pada kelapa sawit.*

[Kata kunci: ACCs, WRI1, TCP4, fosforilasi, asam lemak]

Abstract

Seaweed specifically, *Sargassum* sp. is known to contains a boosting hormone growth that has been promoted plant growth and yield due to the containing of auxin, gibberelic acid (GA) and cytokinine, and also some amino acids especially glutamic acid. Those composition could be used as an booster of palm oil production which related to acetyl co-A carboxylase activity (ACC). ACC is the rate determination step in fatty acid accumulation, and becomes active through dephosphorylation of some serine residues that induced by magnesium and glutamate. Moreover, ACC was regulated by AtWRI1 and AtWRI1-TCP4 interaction, a mechanism that allow fine-tuning of the oil biosynthetic pathway. In this research we conducted gene expression experiments, and molecular docking analyses to study the possible mechanism of seaweed composition stimulating oil accumulation in the oil palm. Further analysis was conducted to ensure

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whether the interaction between TCP4 and candidate inhibitors were able to phosphorylate TCP4 and decrease its activity. GA application resulted in the increase of oil accumulation in 1 month after application, although in the second month the oil accumulation showed decreasing. Increase of oil accumulation in the first month in line with the increase of the expression of ACC in 3rd and 5th weeks. Meanwhile, TCP4 showed decrease expression that resulted in the increase of the WRI1 in 5th week. From this result, it was indicated that GA application could block the TCP4, so it could not interact with WRI1, resulted in the expression of WRI1 and ACC. This interaction stimulates the oil accumulation in oil palm.

[Keywords**:** ACCs, WRI1, TCP4, phosphorylation, fatty acids]

Introduction

Seaweed specifically, *Sargassum* sp. for decades has actually been used directly as a soil conditioner or fertilizers in the world and its extracts has also been widely marketed as a useful additive in plant biofertilizers (Cocozza et al., 2011). *Sargassum* sp. also contains a boosting hormone growth that has been shown to increase plant growth and yield (Ali et al., 2021 & Pradhan et al., 2022) and containing lots of minerals essential from the sea needed by plants as well. Our prior research found that seaweed extract contains some plant hormone such as auxin, gibberelic acid (GA) and cytokinine, and also some amino acids especially glutamic acid (Kresnawaty et al., 2023).

The oil production in oil palm (*Elaeis guineensis*) was determined by acetyl co-A carboxylase (ACC) enzyme, which is the first step in determining the rate of reaction in the fatty acid biosynthetic pathway (Ohlrogge et al., 2018). In human, the catalytic function of acetyl-CoA carboxylase (ACC), is regulated by phosphorylation and dephosphorylation reaction. ACC becomes inactive through phosphorylation of some serine residues (-79, -1,200, and -1,215) by AMP kinase (Hardieet et al., 1997). This enzyme is reactivated through dephosphorylation by activity of phosphatase type 2 (PP2A). Gaussin et al. (1996) concluded that in the cytosol, magnesium and glutamate activate protein phosphatase (GAPP) which removes phosphate groups and activates ACC. While GAPP is actually similar to PP2A due to its sub-cellular distribution and sensitivity to PP2A inhibitors. Kowluru et al. (2001) reported that PP2 phosphatase sensitive to glutamate and magnesium dephosphorylates and activates ACCs on pancreatic beta cells in humans. The same results were obtained by Vavvas et al. (1997) that magnesium and the glutamate-sensitive enzyme PP2A regulate ACC activation in muscle.

ACCs are regulated by transcription factors in oil seeds, including WRINKLED1 (WRI1), and APETALA2 (AP2)-type transcription factor with two AP2 DNA-binding domains (Kong et al., 2020). To understand the role of WRI1 in the regulation of seed oil accumulation and in seed maturation and germination it is important to identify direct targets of WRI1 and its binding site sequences. The AtWRI1-TCP4 interaction is one of the mechanisms that allow fine-tuning of the oil biosynthetic pathway. A recent study showed that TCP4 which similar to AtWRI1, is a target of post-translational modification (Kubota et al., 2017; Kong et al., 2020). However, the potential interactions of AtWRI1 with other TFs in oil biosynthesis have not known yet. TCPs are plant-specific TFs that play important roles in diverse biological processes, such as shoot apical meristem and leaf development, phytohormone biosynthesis, regulation of circadian clock rhythm, and immunity (Liu et al., 2018; Perez et al., 2019). TCPs are known to control plant development, defense, and redox regulation. However, no report so far indicates the involvement of TCPs in regulating oil biosynthesis. TCP4 displayed strong correlation with AtWRI1 during embryo development and that TCP4 negatively affected AtWRI1-stimulated oil biosynthesis. Kobuta et al. (2017) predicted three possible mechanisms by which TCP4 decreases AtWRI1 activity toward its target promoters: (1) the TCP4-AtWRI1 complex occupies a target gene promoter through binding to the TCP- and WRI1-binding *cis*-elements; (2) the complex binds to the promoter only through the WRI1-binding *cis*-element; and (3) TCP4 interaction with AtWRI1 reduces AtWRI1 binding to its binding *cis*-element. In this research we investigated whether ACC in oil palm was also activated with the same mechanism as human ACC. We assumed that the specific compound that could bind with TCP4 in the site of TCP4 binding with WRI1, will increase the expression of WRI1 as well as the expression of ACC that caused higher oil accumulation. The aim of this research was to evaluate GA role in acetyl-coA regulation to improve oil accumulation in oil palm. We hypothesized that GA will increase the oil accumulation and stimulate the gene expressions of WRI1dan ACC, and decrease the TCP4 expression.

Materials and Methods

Materials

Oil palm trees were grown in plot trials area in Ciomas, Bogor, owned by the Indonesian Oil Palm Research Institute (IOPRI), Bogor Unit. The application was formulated using gibberellic acid (GA) with the concentration of 10 and 100 ppm. The treatments were applied by the trunk injection of oil palm trees with 20 ml of 10 ppm GA (B), and 20 ml of 100 ppm GA compared with the control (C). The treatments were applied to 12 years old of oil palm trees, with three of replication, and was repeated every month for 1 week in 2 months. In 1, 3 and 5 weeks after the first treatments, the leaves were taken for analyses of the genes expressions whilst the fruits were taken every month to analyzed the lipid content. The protein sequence of oil palm acetyl-coA carboxylase, Serine/threonine-protein phosphatase and WRI1 deposited in protein data bank and NCBI (https://www.ncbi.nlm. nih.gov/) were analyzed.

Total RNA isolation and cDNA synthesis

The RNA extraction method from fruit and leaf was conducted by following User Manual of RNeasy Mini kit (Qiagen). One gram of tissue was mixed with 1.5% polyvinylpyrrolidone (PVP) 40 and crushed using a pestle and mortar with the addition of liquid nitrogen. After that the mixture was centrifuged, and washed with an equal volume of 70% cold ethanol. Subsequently, ethanol was allowed to evaporate at room temperature for 15 min, and the purified RNA pellet was then resuspended in an appropriate volume of nuclease-free water. The purity and concentration of the RNA was determined spectrophotometrically at ƛ 230, 260, and 280 nm wavelength, while the integrity of total RNA was checked by electrophoresis gel analysis.

Synthesis of cDNA and reverse transcriptase quantitative PCR (RT qPCR) setup

RNA of the palm oil was treated with DNaseI (Fermentas) according to the manufacturer's instructions, and the first-strand cDNA template was synthesized from 1.0 µg of total RNA using the First-Strand cDNA Synthesis Kit (Fermentas) with oligo (dT)18 as the primer, and stored at −20 °C until use the next day. Our experimental processes were consistent for all treatments (Lu et al., 2013).

The analysis using qPCR used a plate with 96 wells which added by qPCR reaction mixture that consisted of 10 µL of SYBR (SYBR Hi-Rox Kit), 1 µL forward primer (10 µM), 1 µL reverse primer (10 µM) (Table 1), 7 µL NFW and 1 µL cDNA from each dilution concentration (100, 50, 20, and 10 ng). The reaction mixture was put into 96 well plates of qPCR (Applied Biosystem StepOne Real Time PCR System) and running for the condition which were: qPCR cycle conditions consist of one denaturation cycle at 95 °C for 10 minutes and followed by 45 amplification cycles (95 °C for 15 seconds, 60 °C for 1 minute and 72 °C for 20 seconds), melt curve stage (95 °C for 15 seconds, 60 °C for 1 minute and 95 °C for 15 seconds) and cooling phase at 4 °C for 30 minutes. Transcript accumulation was calculated automatically by StepOne Software v2.3 provided by the manufacturer using the following calculation:

$$\text{Normalized Ratio} = 2^{-\Delta(\text{Cp target-CpEgActin})}.$$

This form of the equation may be used to compare the gene expression in two different samples; each sample is related to an internal control gene (Actin). To ensure the validity of the data, a stringent threshold of ratio value was applied (Putranto et al., 2015).

Table 1. Primer pairs used in this research
Tabel 1. Pasangan primer yang digunakan dalam penelitian ini

Gene/*Gen*	Sekuens primer/*Primer sequences*
ACCase subunit biotin carboxylase	5'GAAGCACCWTYCCTGCMYT3'
(actin)	3'CKHGTTGGRGCCAYACRAT5'
WRINKLED1	5'CCCGGGTATGAAGAGACCCTTAACCAC3'
	3'GGATCCCGACAGAATAGTTCCAAGAA5'
TCP4	5'CCTCATCCAATCCTTCTCCA3'
	3'GTTGTGGCCAAAGGAAAGAA5'

Analysis of oil content

The mesocarp (fruit flesh) of oil palm was separated from the seeds, cut into small pieces and determined the fresh weight. After an overnight drying (± 16 h) in the oven at 50 °C, the dried samples were weighed and mashed. The samples were then wrapped in filter paper, put in a tube on top of a fat flask containing 20 mL benzene petroleum p.a and mounted on a Soxhlet device. After refluxed for ± 8 h or until the solvent in the Soxhlet looks clear (no more oil was extracted), the fat flask was removed and dried, so that only the oil was left. The flask was then weighed again. The weight of the oil is the weight of the pumpkin after soxhlation minus the weight of the flask before soxhlation.

Template search, model building, and quality assessment

Template search was performed using Phyre2 (http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index) and against the Swiss-model template library (https://swissmodel.expasy.org/). The ACC was searched with BLAST against the amino acid sequence contained in the Protein Data Bank (PDB) using Yeast-specific serine/threonine protein phosphatase (ID PPZ1) of *Candida albicans* (ID 5jpe) as template. The templates with the highest scoring crystal structure (2.61 Å) have then been selected for model building. Models are made according to the target template alignment using Swiss model. The global and per-residue model quality was assessed using the QMEAN scoring function (Hass et al., 2013).

Prediction of ligand binding site

The molecular docking studies of the major constituents on the oil palm protein was performed using PyRx v0.8 with Vina Wizard tool to find out the binding energy and to know the various ligand receptor interactions responsible for the binding affinity of ligands (organic acids). Drug discovery studio (DDS) was used for the visualization of docking process (Chandel et al., 2020).

Results and Discussion

The addition of 10 ppm GA as a stimulator in oil palm production successfully increased oil accumulation in 1 month, while in the second month it showed 10% reduction of oil accumulation. However, at the treatment of 100 ppm GA, oil accumulation increased in the second month, but not significantly different compared to the control (Figure 1). The expression level of ACC increases in the first, third and fifth weeks as shown in Figure 2A. However, GA application resulted in decreasing of TCP4 expression in 1st and 3rd weeks, but the expression was decreased after 5 weeks. The expression of WRI1 showed significantly increased in 5th weeks especially in 10 ppm of GA treatment.

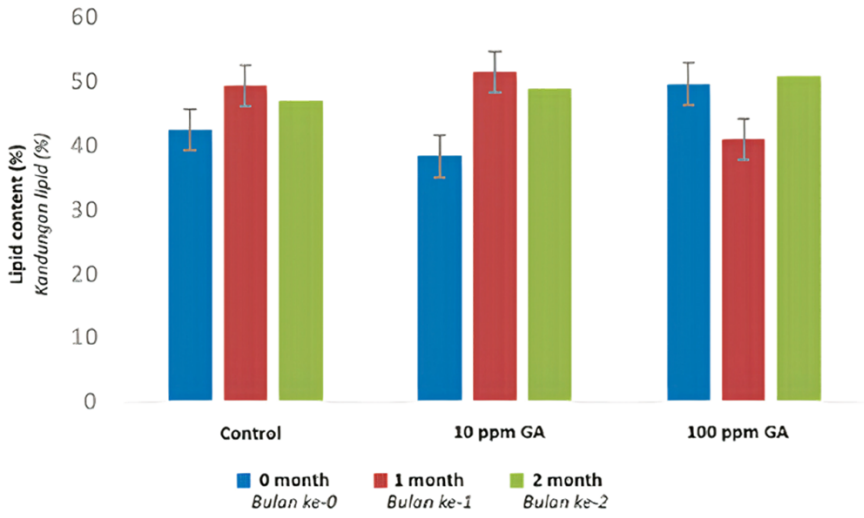

Figure 1. Lipid content of GA treatments in 0,1 and 2 months after treatments
Gambar 1. Kandungan lipid dari perlakuan GA pada 0, 1 dan 2 bulan setelah perlakuan

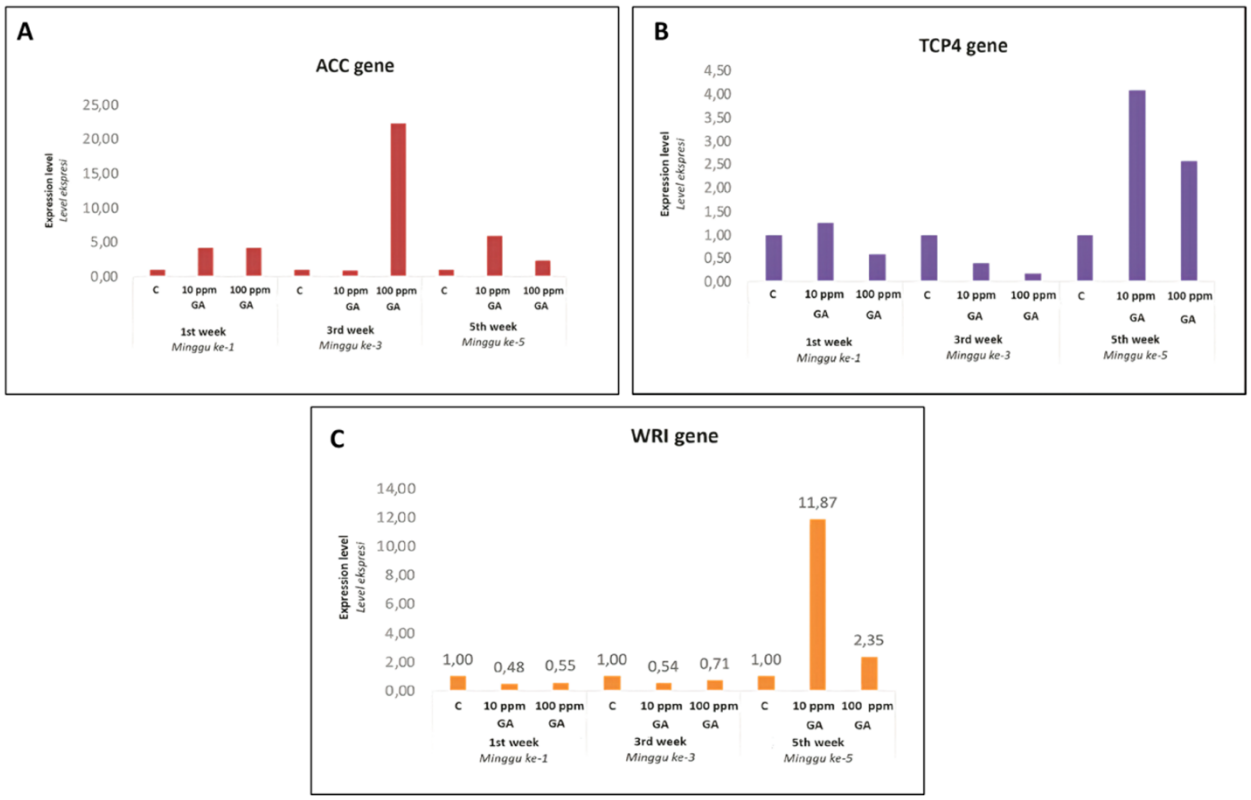

Figure 2. Expression level of ACC (A), TCP4 (B) and WRI1 (C) gene after GA treatments at 1st, 3rd and 5th weeks
Gambar 2. Tingkat ekspresi dari gen ACC (A), TCP4 (B) dan WRI1 (C) setelah perlakuan GA dalam 1, 3 dan 5 minggu

According to Hardie & Carling (1997), the catalytic function of human acetyl-CoA carboxylase (ACC) is controlled by phosphorylation and dephosphorylation processes where ACC becomes inactive due to phosphorylation of serine residues (-79, -1,200, and -1,215) by AMP kinase. By comparing the human ACC amino acid with oil palm ACC, it is known that oil palm has a different sequence from the human ACC (Figure 3). Amino acids no 79, 1,200 and 1215 were found as not serine amino acids. However, serin was found on the order of 66, 1211, 1242, 1244 and 1245. The three-dimensional structure modeling of oil palm ACCase was carried out using the ACC of yeast as the template and obtained GMQE values of 0.59 QMEAN -2.68 and Seq Identity 43.82%. The template of Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform Protein Phosphatase 2A (Aalpha-B56alpha-Calpha) holoenzyme in complex with a small molecule activator of PP2A (SMAP) was used as template for testing the active compound from seaweed extract using molecular docking.

Multiple alignment was conducted to investigate the similarity of ACC protein of oil palm with others species and to find the closest species with high similarity. The result showed that the closest similarity showed by *Saccharomyces cerevisiae* (yeast) (Figure 3). After we obtained the modeling protein of oil palm ACC using template 5jpe from yeast, the molecular docking was conducted to get the best ligand that induced the ACC activity (Table 2) and it showed that citric acid and glutamic were the compounds that had the higher binding affinity which binded with Arginine 261, Arginine 386, Histidine 290, Histidine 231, Histidine 413, and Asparagine 289 (Figure 4).

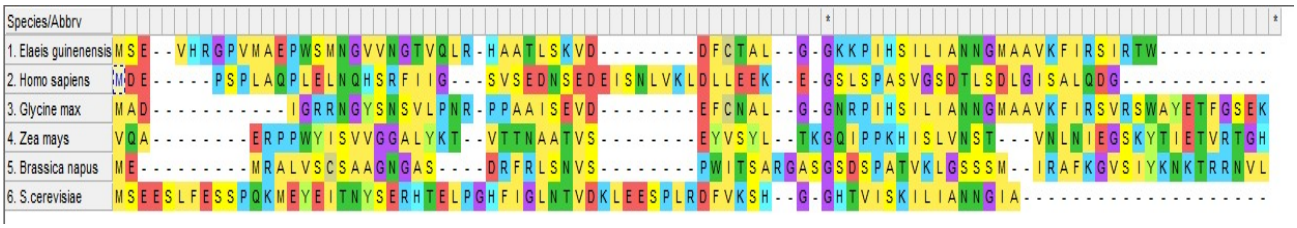

Figure 3. Multiple alignment of oil palm ACC compared with *Homo sapiens*, *Glycine max*, *Zea mays*, *Brassica napus* and *S.cerevisiae*

Gambar 3. Hasil pensejajaran berganda ACC kelapa sawit dibandingkan dengan Homo sapiens, Glycine max, Zea mays, Brassica napus *and* S.cerevisiae

Table 2. Binding affinity of amino acids and organic acid to 5jpe phosphorilase enzyme
Tabel 2. Afinitas ikatan asam amino dan asam organik terhadap enzim fosforilase 5jpe

Receptor *Receptor*	Ligands *Ligan*	Binding affinity *Afinitas ikatan* (kJoule/mol)
Phosphorylase enzyme (ID 5JPE)	Ascorbic acid	-5.8
	Citric acid	-6.1
	flc	-6.0
	Oxalic acid	-4.5
	Succinic acid	-4.9
	Glutamic acid	-5.61
	Asparagine	-5.5
	Glutamine	-4.81
	Lysin	-4.57
	Tryptophan	-4.29

TC4P is regulated by phosphorylation with phosphorylation sites: 4, 13, 41, 60, 61, 68, 84, 87, 100, 104, 133, 134, 137, 138, 141, 167, 180, 184, 187, 193, 194, 195, 198, 199, 200, 210, 212, 213, 214, 222, 225, 238, 241, 256, 258, 274, 275, 276, 292, 302, 306, 310, 313, 314, 318, 341, 342, 343, 345, 349, 354, 383, and 386 of amino acids. Six candidates' inhibitors for phosphorylating TCP4 were identified, including NADH, GA, NADPH, CA, Aspirin, and Coumarin (Table 3). The binding of GA with TCP4 showed hydrogen binding in LYS115, and ALA131 site. In addition, the Van der waals bond were observed in GLU120, PRO130, PRO129, GLY116, ALA132, ASP177, LYS186, HIS113 and Pi-Alkyl: PRO174, LEU176, and ALA182 sites (Figure 5).

Phosphorylase enzyme that involved in the acetyl co-A carboxylase reaction is protein phosphatase 2A (PP2A) which is a serine/threonine phosphatase which has 2 subunits A and B. Furthermore, the bond formed with PP2A enzyme and citric acid was checked whether it could increase the enzyme activity. Docking study was carried out in PP2A phosphatase exhibiting hydrogen bonds with the –OH group of citric acid bound to arginine (261), histidine (290), histidine (413), histidine (231), asparagine (289), and arginine (386). Boone et al. (2000) concluded that glutamate activates the ACC isoform *via* a microcystin-intensive mechanism. It appears that glutamate acts directly as an allosteric activator. Highly pure ACC was observed to cause obstruction of the dephosphorylation process. The 3-dimensional structures of citrate and glutamate can be very closely aligned, including the superposition of the two carboxyl groups. This structural overlap suggests that citrate and glutamate can interact with similar residues in ACC. The fact that glutamate and citrate have a non-additive effect on maximum ACC activity also suggests a similar site and or active mechanism.

Table 3. Binding affinity of candidate compounds with TCP4
Tabel 3. Afinitas ikatan kandidat senyawa dengan TCP4

Ligands *Ligan*	Binding affinity *Afinitas ikatan* (kcal/mol)
Gibberelic acids	-7.2
NADPH	-7.1
Chlorogenic acid	-6.7
Aspirin	-6.6
Coumarin	-6.6
Pro-Hyp	-6.4
tcp4_pdb_644066 (standard ligand)	-6.4
Acyl-CoA	-6.3
Salicylic acid	-6.3
Fulvic acid	-6.3
ATP	-6.2
ABA	-6.1
BAP	-6.0
Sucrose phosphate	-5.8
Acetyl-CoA	-5.8
Sucrose	-5.8
Glucose 6 phosphate pyruvat	-5.7
Gly-Pro-Hyp	-5.6
Paclobutrazol	-5.4

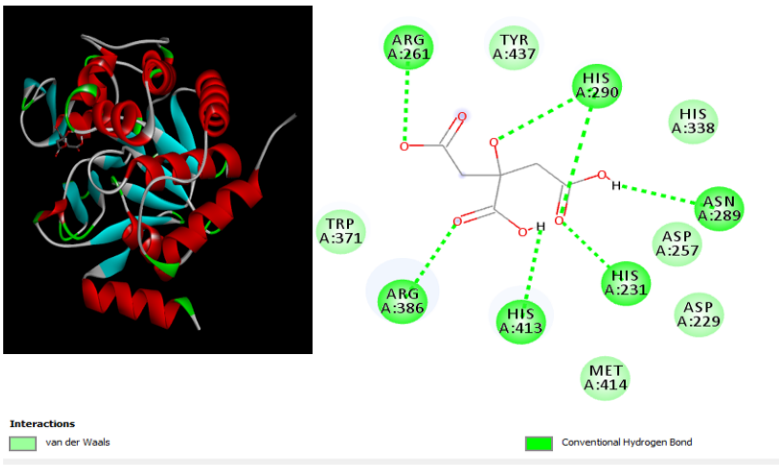

Figure 4. Chemical binding of phosphorylase enzyme PP2A against citric acid
Gambar 4. Ikatan kimia enzim fosforilase PP2A dengan asam sitrat

The inhibition activity was shown by molecule binding site towards phosphorylation sites of TCP4. Further analysis was conducted to ensure whether the interaction between TCP4 and candidate inhibitors were able to phosphorylate TCP4 and decreasing its activity. GA application resulted in the increase of oil accumulation after 1 month of application, although in the second month the oil accumulation shows decreasing. Increase of oil accumulation in the first month in line with the increase of the ACC expression of in 3rd and 5th weeks. On the other hand, TCP4 showed decrease expression that result in the increase of the WRI1 in the 5th week. From this result we hypothesized the possible mechanism as shown in Figure 6. The GA application was assumed could block TCP4, so it could not interact with WRI1 that caused the increase of WRI1 and ACC expression and stimulate the oil accumulation.

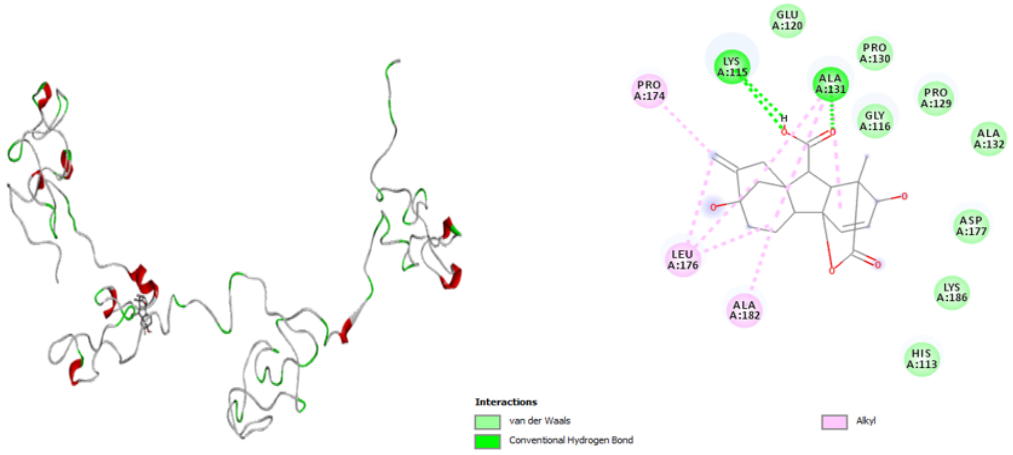

Figure 5. The binding of TCP4 with GA
Gambar 5. Ikatan TCP4 dengan GA

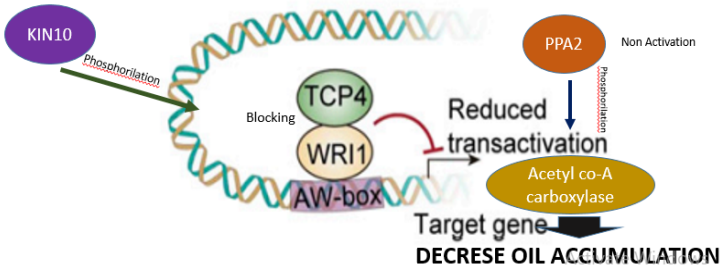

Figure 6. Model of the proposed TCP4-mediated repression of AtWRI1 transactivation (modification of Kong et al., 2020)
Gambar 6. Model yang diperkirakan menjadi mekanisme represi transaktivasi AtWRI1 (modifikasi dari Kong et al., 2020)

Conclusion

The increase of palm oil accumulation in the first month is in line with the upregulation ACC expression in 3rd and 5th weeks after treated with gibberellic acid (GA) from seaweed. However, TCP4 showed down regulation expression and induced WRI1 level in the 5th week. The GA application was assumed blocking TCP4 and disturbing TCP4-WRI1 interaction. This interaction stimulates oil accumulation in the oil palm. Nevertheless, further research should be conducted in the proteomic field to gain additional information of GA inactivation mechanism affecting TCP4 expression.

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