

cyp11A1 CANOLA PLANTS UNDER SHORT TIME HEAT STRESS CONDITIONS

L.O. SAKHNO¹, M.S. SLYVETS^{1,2}, M.V. KUCHUK¹

¹ Institute of Cell Biology and Genetic Engineering NAS of Ukraine, Kyiv
E-mail: sakhno@icbge.org.ua

² National Technical University of Ukraine «Kyiv Polytechnic Institute»

*In order to investigate the high temperature tolerance of spring canola plants (*Brassica napus* L.) constitutively expressing *cyp11A1* gene which encodes bovine cytochrome P450_{SCC} the growth features were analyzed under short time heat stress (42 °C) in growth chamber. Earlier it was documented that results of the heat tolerance test positively correlated with improvement of high temperature resistance in field trial. Higher relative water content (by 13 %) and superoxide dismutase (SOD) activity, lower electrolyte leakage (up 1.4-fold) and smaller increase in chlorophyll a and carotenoid contents in *cyp11A1* canola leaves in comparison with wild-type plants under stress allowed to conclude *cyp11A1* plants are more tolerant to high temperature than the control ones. We suppose that SOD activity increase which revealed in our transgenic canola in normal condition plays the defining role in the biochemical alterations in plant metabolism for the thermotolerance improvement. SOD activity increment could be caused by heterologous cytochrome P450_{SCC} activity which resulted in the superoxide radical formation. *Cyp11A1* canola plants might be resistant to the other stress conditions of different origin.*

Key words: *Brassica napus*, *cyp11A1*, cytochrome P450_{SCC}, photosynthetic pigments, heat stress, SOD.

Introduction. Increasing the oil content, quality and yield remains the major aim of oilseed rape (*Brassica napus* L.) breeding. Resistance to abiotic stresses becomes essential characteristic of plants because of climate changes.

Biotechnological methods are successfully used for understanding plant abiotic stress tolerance [1] and for applying for rapeseed creation with different stress resistance [2–5]. Transgenic canola plants overexpressing a vacuolar Na⁺/H⁺ antiport from *Arabidopsis thaliana* were able to grow, to flower, and to produce seeds in the presence of 200 mM NaCl. Seed yields and seed quality were not affected by the high salt concentration [2]. Overexpression of wheat mitochondrial Mn superoxide dismutase (Mn SOD3.1) enhanced transgenic canola heat, drought and cold tole-

rance both in the field and under artificial stress conditions [3]. Transgenic *B. napus* carrying b-subunit of *Arabidopsis* farnesyltransferase (ERA1) antisense construct driven by a drought-inducible rd29A promoter were more resistant to seed abortion induced by water deficit during flowering [4]. Field trials suggested that with adequate water, transgenic canola produced the same amount of seed as the parental control. Under moderate drought stress conditions at flowering, the seed yield of transgenic plants was significantly higher than the control [4]. Overexpression of the RNA-binding domain of the flowering control locus A protein led to increase in plant size, organ size, cell size, plant productivity, and oil content in transgenic rape plants by down-regulating the cell-cycle-related *cyclin-B2-1* gene, an activator of cyclin-dependent kinase 1 [5]. Transgenic tall fescue (*Lolium arundinaceum* Darbyshire) plants expressing CuZnSOD and ascorbate peroxidase genes in chloroplasts under the control of the oxidative stress-inducible promoter, sweet potato peroxidase anionic 2 (SWPA2), have improved resistance to several abiotic stresses, such as methyl viologen, H₂O₂, and the heavy metals (copper, cadmium, and arsenic) [6].

We have constructed canola lines carrying bovine *cyp11A1* gene in their nuclear genome [7] using *Agrobacterium tumefaciens*-mediated leaf disk transformation [8]. This gene encodes cytochrome P450_{SCC} from bovine adrenal cortex mitochondria and was shown to affect the biosynthesis of steroid compounds in transgenic tobacco (*Nicotiana tabacum* L.) [9]. Obtained canola plants were resistant to BASTA herbicide treatment in greenhouse conditions due to *bar* gene expression. It was used in transformation cassette as a selective marker. Some of transformants accumulated an increased amount of total soluble proteins in leaves and seeds. They have enhanced antioxidant activity in leaf tissues. Some of them flowered 5–7 days ear-

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lier than the control plants. Changes in fatty acid composition of leaf lipids were detected by using gas chromatography [10]. We have shown that the integration of *cyp11A1* gene of animal origin under constitutive (35S) promoter also affected canola oil composition. Increase in oleic acid (from 66 to 73 mole%) was accompanied by decrease in linolenic (from 6 to 3 mole%) acid. The total fatty acid content in canola seeds remained at the level of control plants. Superoxide dismutase (SOD) activity in transgenic *cyp11A1* canola leaves was higher than in the control ones on ~30 % in optimal aseptic conditions. Plant testing for *in vitro* osmotic stress resistance made it possible to identify the line that could generate biomass under hyperstress comparable with the control one in the normal conditions [11]. Analysis of seed germination under high temperature revealed differences between control and *cyp11A1* seedlings in fresh weight, hypocotyl and root length, SOD activity [12].

A number of changes mentioned for *cyp11A1* canola plants are inherent in the plants with heterologous SOD expression, which are characterized by increasing adaptive properties when they were subjected to stresses of various origins [3, 13]. And the aim of the present work was the investigation of *cyp11A1* canola thermotolerance by using the short time heat stress. It was documented that results of the heat tolerance test (short-time growth under 42 °C in growth chamber) positively correlated with improvement of high temperature resistance in field trial [3]. We analyzed such physiological and biochemical parameters which are usually used to characterize the properties of plants under stress as relative water content [14, 15], electrolyte leakage [15, 16], photosynthetic pigment content and ratios [15, 17–19], SOD activity [3, 20, 21].

Materials and methods. Spring canola plants (*Brassica napus* L.) cv Mariia (National Agrarian University of the Ukrainian Academy of Agrarian Sciences selection) were used as the control plants because earlier they were used for primary *cyp11A1* transformant creation [7]. Transgenic homozygous T₂1a and T₂2c lines which were obtained by self-pollination of primary transformants under greenhouse conditions [7] (T₂ generation) were also analysed. They were selected as the most tolerant to osmotic stress in previous experiments under *in vitro* conditions [11]. Aseptic plants were transplanted into soil in greenhouse (12/12 photoperiod,

+23 °C). After two weeks they were transferred into Programmable Plant Growth Chamber, model WGC-P9 (WiseCube®WGC, Korea).

Heat tolerance tests were conducted after two week chamber growth under the following conditions: 16 h (light)/8 h (dark) photoperiod, temperature +22 °C (day)/+18 °C (night), 70 % humidity, 480–550 μmol/m²s light intensity). Humidity and light intensity were taken without changes. Temperature was increased with 2 °C/h gradient to 42 °C. Plants were than taken isothermal at 42 °C for 16 h [3].

SOD activity was measured by using photochemical oxidation of nitro blue tetrazolium method [22]. Fresh plant material (100 mg) in Eppendorf tube (1.5 ml) was rubbed with 1 ml of 50 mM Tris-HCl buffer (pH 8.0) and was centrifuged at 13 000 g (4 °C) for 15 min. The supernatant was used for analyses. Reaction was held in Eppendorf tube (1.5 ml). One tube for each probe was retained in the dark. The others were illuminated with white light lamp (fluorescent lamp T5/G5, model ELI-230A-T5-8W) during 5 min in thermostat at 23 °C. The optical density of illuminated probe solution was measured at 550 nm (BioPhotometer Eppendorf, Germany) versus optical density of dark probe. Null probe had no leaf extract in its composition. SOD activity was expressed in relative unit/mg protein.

The total soluble protein content was measured using Bradford method [23].

Relative water content (RWC) was calculated as

$$\text{RWC, \%} = 100 \cdot (\text{FW} - \text{DW}) / (\text{TW} - \text{DW}), \quad (1)$$

FW – fresh weight of leaf discs (2 cm diameter) immediately after sampling; TW – turgor weight the same discs after saturation during 24 h in Petri dish with deionized water; DW – dry weight the same discs after lyophilisation.

Membrane permeability was estimated by recording electrolyte leakage (EL) [24]. Fresh leaf discs (diameter 2 cm) were washed thrice with deionized water to remove adhered electrolytes. Then the samples were placed in the closed vials containing 20 ml deionized water. They were incubated at 25 °C on rotary shaker for 24 h. Electrical conductivity of the solutions (L_1) were subsequently determined on electroconductometer CyberScan pC510 (Eutech Instruments, Germany). Then the samples were autoclaved at 120 °C during 20 min

and the final electrical conductivity (L_2) was obtained after equilibration at 25 °C. The EL was defined as follows

$$EL (\%) = (L_1/L_2) \cdot 100. \quad (2)$$

Photosynthetic pigment determination was conducted according Wellburn [25] with dimethyl sulfoxide as a solvent (Dimethyl sulfoxide extra pure, «Merck»). Leaf sample (50 mg) was placed in a vial with 4 ml of the reagent and incubated in a water bath at 67 °C during 4 h. The extract absorbance was measured at 665, 649, and 480 nm on spectrofluorimeter «Phluorat-02-Panorama» (Lumex-Marketing, Russia). The calculation was carried out by the formulas:

$$C_a = 12.19A_{665} - 3.45A_{649}, \quad (3)$$

$$C_b = 21.99A_{649} - 5.32A_{665}, \quad (4)$$

$$C_{x+c} = (1000A_{480} - 2.14C_a - 70.16 C_b)/220, \quad (5)$$

C_a , C_b , C_{x+c} – content of chlorophyll *a*, chlorophyll *b*, and total carotenoids, respectively.

Statistical analysis was performed according to Duncan multiple range test. Differences from control values were significant at $p \leq 0.05$. Three independent experiments were conducted in five replications. There were three replications for formazan measurement for each extract.

Results and discussion. High-temperature stress is defined as the rise in temperature beyond a critical threshold for a period of time sufficient to cause an irreversible damage to plant growth and development [26]. High leaf temperatures reduce plant growth and limit crop yields.

Earlier we documented that SOD activity in transgenic *cyp11A1* canola leaves was higher than in the control ones in the optimal aseptic conditions [7, 27]. In growth chamber it was up 1.76-fold higher comparable with control at 22 °C (Fig. 1). Cytochrome P450_{SCC} catalyzes three steps cholesterol oxidation with formation of pregnenolone in animals [28]. Superoxide radicals are formed during these reactions. SOD activity in *cyp11A1* canola can be increased due to cytochrome P450_{SCC} activity. It did not change under short time heat stress in *cyp11A1* leaves (Fig. 1). SOD activity increased in control by 18 % but it retained lower in comparison with in transgenic ones.

In our experiments of *in vitro* *cyp11A1* growth under osmotic stress we demonstrated that SOD

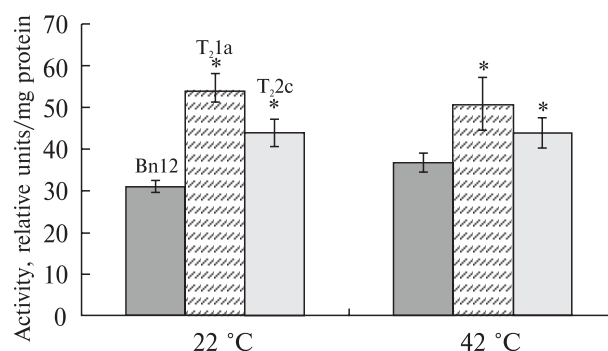


Fig. 1. SOD activity in canola leaves before (growth temperature 22 °C) and after (temperature 42 °C) heat stress: Bn12 are control plants (cv Mariia), T₂1a and T₂2c are homozygous *cyp11A1* lines of second generation. Here and in Fig. 2, 3 error bars represent mean \pm one standard deviation and asterisks * indicates significant differences between experimental values compared with the control ones ($p \leq 0.05$)

activity of T₂1a line was higher than the control one in the absence of stress and was unchanged at 100 mM mannitol addition to the culture medium [11]. After mannitol increasing in the medium up to 200 mM SOD activity began to rise markedly in T₂1a line and was maximal in control. It continued to rise at 500 mM mannitol in T₂1a, while it decreased in control. SOD activity of *cyp11A1* plants increase in normal conditions allows them not to take deteriorating conditions as stress and maintains for prolonged period the unchanged physiological and biochemical characteristics.

It was shown that the innate threshold temperature was dependent upon endogenous SOD and glutathione reductase activity in *Gossypium hirsutum* plants [20]. Under identical growth conditions, thermotolerant *G. hirsutum* plants had significantly higher optimal and threshold temperatures for the actual quantum yield response of photosystem II and glutathione reductase activity than thermosensitive ones. The authors concluded that maintaining a sufficient antioxidant enzyme pool prior to heat stress is the mechanism for coping with rapid leaf temperature increases that commonly occur under field conditions. Heat stress also increased SOD activity in wild type *Nicotiana tabacum* plants but this increase was much greater in transgenic plants expressing betaine aldehyde dehydrogenase [29]. The increased thermotolerance induced by accumulation of glycinebetaine was associated with the enhancement of the repair of PSII from heat-

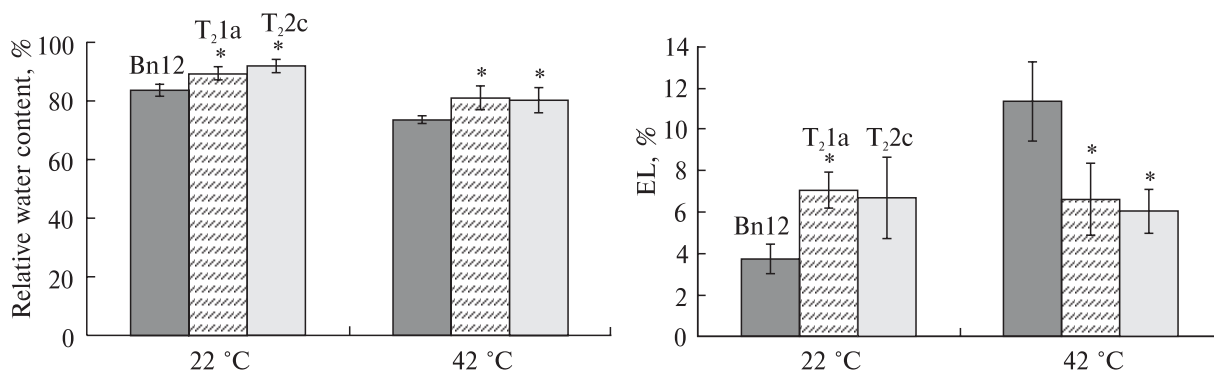


Fig. 2. Relative water content (a) and electrolyte leakage (b) in canola leaves before (22 °C) and after (42 °C) short time heat stress: Bn12 are control plants (cv Mariia), T₂1a and T₂2c are homozygous *cyp11A1* lines of second generation

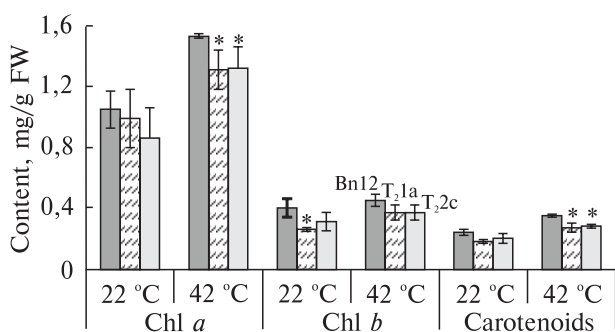


Fig. 3. Photosynthetic pigment content in canola leaves before (growth temperature 22 °C) and after (temperature 42 °C) heat stress: Bn12 are control plants (cv Mariia), T₂1a and T₂2c are homozygous *cyp11A1* lines of second generation

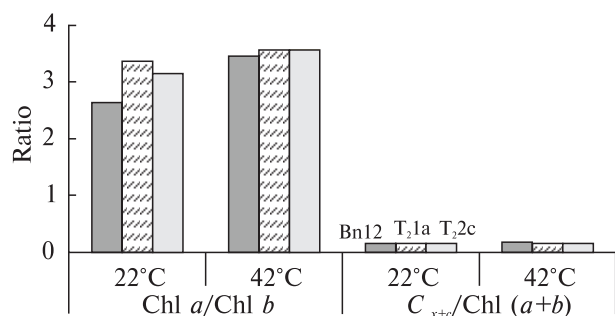


Fig. 4. Photosynthetic pigment ratios in canola leaves before (growth temperature 22 °C) and after (temperature 42 °C) heat stress: Bn12 are control plants (cv Mariia), T₂1a and T₂2c are homozygous *cyp11A1* lines of second generation

enhanced photoinhibition, which might be due to less accumulation of reactive oxygen species in transgenic plants.

We found that the temperature increase led to RWC reduction in all plant tested on 10–13 % (Fig. 2, a). Nevertheless transgenic plants had significantly higher RWC under high-temperature stress. RWC also decreased with prolonged drought, heat, and combined stresses for tall fescue and Kentucky bluegrass (*Poa pratensis* L.) but the severity of decline varied with stress type and duration [30].

Membrane permeability is an early change indicator of plant physiological functions. It can be used as a criterion of plant tissue resistance to stressors [31]. Conductance-measuring method is one of the most effective and fast way to evaluate degree of cell membrane damage. Analysis of electrolyte leakage from leaf canola tissues revealed differences between the control and transgenic lines (Fig. 2, b). Transgenic plants were characterized by ~40 % increased electrolyte leakage under 22 °C in comparison with control ones. This may indicate that this temperature was not optimal for *cyp11A1* canola. Electrolyte leakage increased significantly (up 2.5 fold) in control plants under heat stress. But it retained without changes in transgenic lines (Fig. 2, b). It testifies the transgenic canola tolerance to short time high temperature. Increased resistance to heat stress was also shown in transgenic canola plants which overexpressed wheat mitochondrial Mn SOD [3]. Electrolyte leakage in most of these plants was lower than in control under high temperature at different growth phases. In chickpea (*Cicer arietinum* L.) a heat stress investigation has shown that membrane stability significantly correlated ($R^2 = 0.7$) with photosynthetic quantum yield and proved to be viable screening technique

for thermotolerance [32]. Rice plants expressing MnSOD from pea (*Pisum sativum* L.) under the control of an oxidative stress-inducible *SWPA2* promoter in chloroplasts demonstrated reduced electrolyte leakage compared to wild type leaf slices and exhibited less injury, measured by net photosynthetic rate, under drought stress induced by polyethylene glycol 6000 [33].

Chl *a* content did not differ in the control and transgenic plants under normal temperature (Fig. 3). It increased at short time heat significantly both in the wild-type and *cyp11A1* canola, but this increase was greater in control plants. Chl *b* content was significantly lower in T₂1a line in comparison with the control and T₂2c plants at 22 °C growth (0.26 ± 0.01 , 0.4 ± 0.06 and 0.31 ± 0.05 mg/g fresh weight, respectively). Under heat it did not change in control and T₂2c plants and increased in T₂1a plants at control level. Carotenoid content was higher in control leaves (0.24 ± 0.02 mg/g fresh weight) versus transgenic plants (0.18 ± 0.01 and 0.2 ± 0.03 for T₂1a and T₂2c plants, respectively) at initial conditions. After 42 °C it increased significantly in all canola but this increase was greater in control plants.

Chl *a*/Chl *b* ratio was lower in the control plants (2.63) under 22 °C (Fig. 4) in comparison with transgenic (3.36 and 3.14 for T₂1a and T₂2c plants). Heat affected the increase in it for all plants up 3.45–3.57. Carotenoids/Chl (*a* + *b*) ratio was unchanged both at 22 and 42 °C for the control as well as *cyp11A1* canola (Fig. 4). The study of interrelation between chlorophyll content and heat and/or high photon flux density has shown that the very low chlorophyll content in Syrian barley landrace decreases leaf absorbance which, in turn, reduces the potentially damaging heating effect of high solar radiation in droughted plants [17]. Chl *b* content retained without changes except for the increase in transgenic T₂1a line at the control level under heat. Carotenoids/Chl (*a* + *b*) ratio was unchanged both at 22 and 42 °C for the control as well as *cyp11A1* canola. Both chlorophylls and carotenoids also accumulated during acclimation to heat (39 °C) in barley cv Plaisant, leading to an almost constant carotenoid:chlorophyll ratio [17]. Since it was found that membrane stability significantly correlated ($R^2 = 0.7$) with photosynthetic quantum yield under heat [32], we can assume the im-

provement of photosynthesis in *cyp11A1* canola in comparison with wild type plants because electrolyte leakage was greater in the latter.

Thus, *cyp11A1* constitutive expression proved the higher relative water content in the leaves of transgenic plants in comparison with the control ones under heat stress in growth chamber. Superoxide dismutase activity in transgenic leaves was up 76 % higher comparable with control at 22 °C and remained unchanged under heat. Electrolyte leakage was lowered. Chl *a* and carotenoid contents were increased under stress in the control as well as *cyp11A1* canola, but they were lower in the latter. Chl *b* content retained without changes except for the increase in transgenic T₂1a line at the control level. So it is possible to conclude that *cyp11A1* plants can be more tolerant to high temperatures than control ones. We suppose that SOD activity increase which revealed in our transgenic canola plays the defining role in the biochemical alteration in plant metabolism allowing heat stress resistance improvement. SOD activity increase could be caused by cytochrome P450_{SCC} activity which resulted in the superoxide radical formation. Manifestation of bovine *cyp11A1* expression in transgenic canola is the same as heterologous *sod* overexpression. Plants expressing *cyp11A1* gene might be resistant to the other stress conditions both of abiotic and biotic origin.

РАСТЕНИЯ РАПСА С ТРАНСГЕНОМ *cyp11A1* В УСЛОВИЯХ КРАТКОВРЕМЕННОГО ВЫСОКОТЕМПЕРАТУРНОГО СТРЕССА

Л.А. Сахно, М.С. Сливей, Н.В. Кучук

Для исследования устойчивости трансгенных растений ярового рапса (*Brassica napus* L.), конститутивно экспрессирующих ген *cyp11A1*, который кодирует цитохром P450_{SCC} быка, к высоким температурам были проанализированы особенности роста в условиях кратковременного высокотемпературного стресса (42 °C) в климакамере. Результаты этого теста положительно коррелируют с повышением толерантности к действию высоких температур в полевых испытаниях. Увеличение относительного содержания воды (на 13 %) и активности супероксиддисмутазы (СОД), пониженный выход электролитов (в 1,4 раза) и меньшее повышение содержания хлорофилла *a* и каротиноидов в листьях трансгенных растений по сравнению с растениями дикого типа в условиях высокотемпературного стресса позволяют сделать вывод о том, что термоустойчивость *cyp11A1* рас-

тений рапса выше, чем контрольных. Увеличение активности СОД, обнаруженное в наших трансгенных растениях рапса в нормальных условиях, играет предположительно определяющую роль в биохимических изменениях растительного метаболизма, что приводит к возрастанию устойчивости к повышенным температурам. Возрастание активности СОД возможно за счет экспрессии гетерологического цитохрома P450_{SCC}, которая приводит к образованию супероксид радикалов. Полученные растения могут быть устойчивы к другим стрессовым воздействиям различного происхождения.

РОСЛИНИ РІПАКУ З ТРАНСГЕНОМ *cyp11A1* ЗА УМОВ КОРОТКОТРИВАЛОГО ВИСОКОТЕМПЕРАТУРНОГО СТРЕСУ

Л.О. Сахно, М.С. Сливець, М.В. Кучук

Для вивчення стійкості трансгенних рослин ярого ріпаку (*Brassica napus* L.), які конститутивно експресують ген *cyp11A1*, що кодує цитохром P450_{SCC} великої рогатої худоби, до дії високих температур проаналізовано особливості росту за умов короткотривалого високотемпературного стресу (42 °C) в клімакамері. Результати цього тесту позитивно корелюють з підвищенням толерантності до дії високих температур в польових дослідах. На підставі збільшеного відносного вмісту води (на 13 %) і підвищеної активності СОД, а також зменшеного виходу електролітів (в 1,4 раза) і меншого підвищення вмісту хлорофілу *a* і каротиноїдів в листках трансгенних рослин у порівнянні з рослинами дикого типу за умов стресу можна зробити висновок про підвищення термостійкості у рослин ріпаку з трансгеном *cyp11A1* порівняно з контролем. Збільшення активності СОД в наших трансгенних рослинах ріпаку, виявлене за нормальних умов, відіграє визначальну роль у біохімічних змінах рослинного метаболізму, що спричиняє зростання стійкості до підвищених температур. Зростання активності СОД можливо за рахунок активності цитохрому P450_{SCC} в результаті якої відбувається утворення супероксид радикалів. Отримані рослини можуть бути стійкими до інших стресових впливів різного походження.

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