



Research article

Saccharomyces cerevisiae cells lacking transcription factors Skn7 or Yap1 exhibit different susceptibility to cyanidin



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ABSTRACT

Anthocyanidins – the aglycone moiety of anthocyanins – are responsible for the antioxidant traits and for many of the health benefits brought by the consumption of anthocyanin-rich foods, but whether excessive anthocyanidins are deleterious to living organisms is still a matter of debate. In the present study we used the model eukaryotic microorganism *Saccharomyces cerevisiae* to evaluate the potential toxicity of cyanidin, one of the most prevalent anthocyanidins found in berries, grapes, purple vegetables, and red wine. We found that yeast cells lacking the transcription factors responsible for regulating the response to oxidative stress – Skn7 and Yap1 – exhibited different sensitivities to cyanidin. Cells lacking the transcription factor Skn7 were sensitive to low concentrations of cyanidin, a trait that was augmented by exposure to visible light, notably blue or green light. In contrast, the growth of yeast cells devoid of Yap1 was stimulated by low concentrations, but it was impaired by high cyanidin exposure. High, but not low cyanidin was shown to induce Yap1 translocation from cytosol to nucleus, probably by generating reactive oxygen species such as H₂O₂. Taken together, these observation suggested that Skn7 and Yap1 have complementary roles in adaptation to cyanidin stress, with Skn7 involved in adaptation to low concentrations and with Yap1 responsible for adaptation to high concentrations of cyanidin. The results imply that caution is needed when utilizing cyanidin-enriched supplements, especially when in combination with prolonged exposure to visible light.

1. Introduction

Fruits and vegetables represent safe sources of natural antioxidants, which are highly recommended for human diet. In recent years, the health benefits brought by plant antioxidants have been so intensively studied and mediatized that they have become important ingredients of the multitude of food supplements available on the market. While the health benefits brought by the daily intake of natural antioxidants are undeniable, there is increasing evidence that excessive consumption needs to be regarded with caution, especially when it comes to dietary supplements which are exceptionally rich in pure compounds (Poljsak and Milisav 2012; Ames et al., 2018; Yang et al., 2018). Anthocyanins are a subclass of polyphenolic phytochemicals with antioxidant traits, thought to be responsible for many of the health benefits associated with the consumption of berries, grapes, currants, red wine, etc. (Smeriglio et al., 2016; Wallace et al., 2016; Jamar et al., 2017; Lee et al., 2017; Lin et al., 2017; Sandoval-Ramírez et al., 2018; Manolescu et al., 2019). Anthocyanins also gathered much attention due to their potential use in food and pharmaceutical industries as safe dyes, stabilizers, functional

foods, nutraceuticals, or food supplements (Khoo et al., 2017). Anthocyanins are glycosides of anthocyanidins, the latter being the true bearer of the biologic activity. Cyanidin is one of the most prevailing anthocyanidins and a major pigment in berries, grapes and purple-coloured vegetables (Wallace and Giusti 2015; Khoo et al., 2017). The studies on anthocyanins deal mainly with their chemoprotective action owed to the antioxidant capacity which is conferred by the anthocyanidin moiety (Tedesco et al., 2001; Heo and Lee 2005; Isaak et al., 2017) and it is widely believed that the toxicity of anthocyanins is very low (Wallace and Giusti 2015). Studies on the potential toxicity of anthocyanins/anthocyanidins are less represented, although some alarm has been triggered concerning their role in the so called ‘antioxidative stress’, which can be correlated with overconsumption of natural antioxidants, either in complex mixtures or as single isolated compounds (Poljsak and Milisav 2012; Kwee 2014; Cai et al., 2018; Cásedas et al., 2019). Although it is considered that the ingestion of an isolated natural compound carries less risk comparing with a complex natural mixture, not many isolated polyphenols have been subjected to rigorous testing (Cásedas et al., 2019). We therefore decided to investigate the potential toxicity of pure

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cyanidin on *Saccharomyces cerevisiae* cells, and while no apparent toxicity was recorded on the wild type, it was noted that the cells devoid of the transcription factors Skn7 or Yap1 exhibited different sensitivity to cyanidin exposure.

S. cerevisiae represents a very good eukaryotic model for the investigation of the cellular response to various antioxidants (Eleutherio et al., 2018; Zimmermann et al., 2018; Lingua et al., 2019) as this microorganism responds to oxidative stress similarly to higher organisms, including humans (Jamieson 1998), at the same time allowing for simplification of the biological context by eliminating redundancies or by overcoming ethical and experimental constraints. Although not numerous, there are studies on *S. cerevisiae* highlighting conditions under which widely used antioxidants such as epigallocatechin-*O*-gallate (Maeta et al., 2007; Mitrica et al., 2012; Ruta et al., 2018) or resveratrol (Madrigal-Perez et al., 2016; Orlandi et al., 2017) are not entirely beneficial.

Yap1 and Skn7 are the main *S. cerevisiae* transcription factors which orchestrate the response to oxidative stress in yeast cells. While association of the two transcription factors has been demonstrated (Lee et al., 1999; Mulford and Fassler 2011), there is evidence that Yap1 and Skn7 also regulate their separate set of genes (Lee et al., 1999). In this study we found that cells lacking Yap1 or Skn7 showed different susceptibility to cyanidin and that the sensitivity of *skn7Δ* cells could be overcome by Yap1 activation upon high cyanidin exposure.

2. Materials and methods

2.1. Reagents and growth media

Unless otherwise specified, all reagents used were purchased from Merck (Darmstadt, Germany). Yeast strains were manipulated as described (Sherman 2002) using standard media YPD (1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose) or synthetic complete SC (0.67 % w/v yeast nitrogen base with (NH₄)₂SO₄, 2% w/v glucose, supplemented with the necessary amino acids). For solid media, 2% w/v agar was used. Cyanidin (Sigma-Aldrich) was used from a stock solution (10 mg/mL in 70% v/v ethanol) sterilized by filtration.

2.2. Yeast strains and storage

The *S. cerevisiae* strains used in this study had the wild-type (WT) background of BY4741 (*MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*). The single-gene deletion strains used were Y02900 (BY4741, *skn7::kanMX4*, denoted *skn7Δ*) and Y00569 (BY4741, *yap1::kanMX4*, denoted *yap1Δ*). The strains were obtained from EUROSCARF (www.euroscarf.de) and stored at -85 °C as 50% glycerol stocks, until recovered for use. Double knock-out strain *skn7Δ yap1Δ* (BY4741, *skn7::kanMX4*; *yap1::kanMX4*) was obtained by crossing of strains Y02900 and Y10569 (*MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*; *yap1::kanMX4*, EUROSCARF) followed by diploid sporulation and random spore analysis (Sprague, 1991).

2.3. Cell exposure to light

Cells to be exposed to light were treated as described (Farcasanu et al., 2013) with slight modifications. Exponentially growing cells were shifted to liquid SC and grown to 10⁷ cells/mL. Viability was checked, and only cells from populations with viability > 99% were shifted to multi-well transparent plates (Nunc ThermoFisher Scientific, approximately 10⁶ cells/well). Cells gently shaken (30–50 rpm) were illuminated from above with light emitting diodes (LEDs) of 100 lumens mounted on a copper ventilated radiator. Cells were illuminated for 2 h with blue monochromatic light (BL, λ = 455 nm), green monochromatic light (GL, λ = 527 nm), or continuous visible spectrum (WL, white light) with intensity on the target up to 105 Lx, determined with a digital Luxmeter LX-1102, Lutron (14.64 mW/cm²).

2.4. Growth assessment of the yeast strains

2.4.1. Growth in liquid media

Overnight pre-cultures were inoculated in fresh medium at density 2 × 10⁵ cells/mL, then incubated for 2 h (the time required for cell adaptation to fresh medium) before cyanidin was added from a sterile stock. The cell density was monitored at by determining the turbidity of cellular suspension at 600 nm (OD₆₀₀). Relative growth was calculated for each culture sample as the ratio between OD₆₀₀ at a specified time and OD₆₀₀ at time 0 (time when cyanidin was added to the medium). Cells in liquid cultures were incubated with shaking (30 °C, 200 rpm) on a multi-amplitude orbital shaking incubator (Thermo Max Q 4000, Thermo Fisher Scientific).

2.4.2. Cell viability

The cell viability, expressed as percentage of live cells within a whole population, was assessed by staining with methylene blue (Roth, Germany) (Kwolek-Mirek and Zdrag-Tecza 2014). Viability was examined microscopically (Olympus BX53, Japan) for at least 300 cells from one biological replicate. Viability was also tested by determining colony forming units (CFU): thoroughly suspended cell samples were suitably diluted with sterile deionized water and plated on YPD/agar. After 3–4 days of incubation at 28 °C, the colonies were counted and viability was expressed as percent of CFU relatively number of initial cells (Kwolek-Mirek and Zdrag-Tecza 2014). When compared, the two methods yielded similar results.

2.5. Plasmids and yeast transformation

The Yap1-GFP (Yap1 tagged at the N-terminus with green fluorescent protein, GFP) was expressed from a plasmid constructed on the pRSII325 vector (Chee and Haase 2012). Plasmid pRSII25 (*LEU2*, high-copy) was a gift from Steven Haase (Addgene, plasmid #35467; <http://n2t.net/addgene:35467>; RRID:Addgene_35467). *YAP1* coding sequence along with 520 bp upstream sequence was amplified using BY4741 genomic DNA as template. The primers used for amplification were: forward, 5'- tacgtc-gacACTCTCGTCTCTCGAAA-3' (introducing *Sa*I site, underlined) and reverse, 5'- tacgaattcttaagcttGTTTCATATGCTTATTCAAAG introducing both *Eco*RI (underlined sequence) and *Hind*III (italic underlined sequence) sites. The *Hind*III site was situated upstream the STOP codon (bold) to allow for GFP fusion. The purified amplicon was restriction-cut and introduced into the *Sa*I-*Eco*RI sites of pRSII325, yielding pRSII325-YAP1. The GFP open reading frame lacking the START codon was amplified from plasmid pGREG600 (EUROSCARF) using forward primer 5'- tacaagcttAGTAAAGGAGAAGAACTTTTCA-3' (introducing *Hind*III site, underlined sequence) and reverse primer 5'- tatactaggt-taTTTGTATAGTTCATCCATG (introducing *Spe*I site, underlined sequence, and STOP codon, bold). The GFP amplicon was introduced in the *Hind*III-*Spe*I sites of pRSII325-Yap1 to yield pRSII325-Yap1-GFP. The plasmid p415-TEF-roGFP2-Tsa2ΔCR (Morgan et al., 2016) was a gift from Tobias Dick (Addgene, plasmid #83239; <http://n2t.net/addgene:83239>; RRID:Addgene_83239). Yeast transformation (Dohmen et al., 1991) was performed using S.c. EasyComp™ Transformation Kit (Invitrogen, Carlsbad CA, USA) following manufacturer's indication.

2.6. Cyanidin assay

Cyanidin quantitation was done with Folin-Ciocalteu reagent (Singleton et al., 1999) as described (Oprea et al., 2014), with slight modifications. To determine cyanidin uptake, approximately 10⁹ cells exposed to cyanidin (pre-washed 2 times) were suspended in 100 μL 10% trion-X100 in 10 mM 2-(N-morpholino)ethanesulfonic acid (MES)-Tris, pH 6, and heated for 2 min at 60 °C. The suspension was diluted 10 times with ethanol (70% final ethanol concentration) and vortexed thoroughly for cyanidin release. After centrifugation (10,000 rpm, 1 min), the

supernatant was used for cyanidin assay against a calibration curve. Accumulated cyanidin was normalized as ng/10⁸ cells.

2.7. Fluorescence measurement of roGFP2-Tsa2ΔCR sensor oxidation

The redox-responsive peroxiredoxin-based probe roGFP2-Tsa2ΔCR was used to measure the relative cell oxidative state caused by H₂O₂ variation. This probe is sensitive enough to monitor the variations in intracellular H₂O₂ based on the fluorescence signal of the cells expressing roGFP2-Tsa2ΔCR (Morgan et al., 2016). Cells in late logarithmic growth phase transformed with p415-TEF-roGFP-Tsa2ΔCR were harvested, washed and re-suspended in 10 mM MES-Tris buffer, pH 6.0, to OD₆₀₀ = 5. Subsequently, 200 μL suspensions (approx. 10⁷ cells/sample) were transferred to flat-bottomed 96-well imaging plates (Nunc™ F96 MicroWell™ Black, Thermo Scientific) using a Thermo Scientific Variskan Flash spectral scanning multimode reader. The fluorescence of the cells expressing roGFP-Tsa2ΔCR was recorded at 510 nm using 2 excitation wavelengths 405 nm and 488 nm (Morgan et al., 2016). To obtain the fully oxidized and fully reduced probes, cells were treated with 20 mM diamide (N,N,N',N'-tetramethylazodicarboxamide) or 100 mM DTT (dithiothreitol) for 30 min, respectively. These samples serve as fully oxidized and fully reduced sensor controls and allow the determination of the degree of sensor oxidation (OxD), calculated as described (Meyer and Dick 2010).

2.8. Yap1-GFP cellular localization

For the detection of Yap1-GFP fluorescence, the cells transformed with pRSII325-Yap1-GFP were grown overnight in SC-Leu, diluted 20 times in fresh SC-Leu and grown for 2 h before cyanidin was added from a sterile stock. Following 2–4 h of cyanidin exposure, live cells were

examined with an Olympus fluorescent microscope system (Olympus BX53, Japan) equipped with a HBO-100 mercury lamp and an Olympus DP73 camera. To detect the GFP signals, a GFP filter set (excitation filter 460–480, dichromatic mirror 585, emission filter 495–540) was used. Nuclei were stained with 1 μg/mL 4',6-diamidino-2-phenylindole (DAPI) and observed using DAPI filter set (excitation filter 340–390, dichromatic mirror 410, emission filter 420). The microscopic photographs were processed by CellSens Dimension V1 imaging software (Olympus, Japan). For each strain, one representative image is shown.

2.9. Reproducibility of the results and statistics

All experiments were repeated at least three times. For each individual measurement values were expressed as the mean ± standard deviation (SD) of duplicate determinations on biological triplicates. The data were examined by analysis of variance with multiple comparisons (ANOVA) using the statistical software Prism version 6.05 for Windows (GraphPad Software, La Jolla, CA, USA). The differences were considered to be significant when $p < 0.05$.

3. Results and discussion

3.1. Yeast cells lacking Skn7 or Yap1 are sensitive to cyanidin

Starting from the idea that – like many other antioxidants – cyanidin may also act as a pro-oxidant (Eghbaliferiz and Iranshahi 2016), we used the eukaryote model microorganism *S. cerevisiae* to evaluate cyanidin toxicity, both on wild type (WT) but also on cells lacking either of the two transcription factors which orchestrate the cell response to oxidative stress, Skn7 and Yap1. It was noticed that while cyanidin showed no apparent toxicity towards WT, long exposure to

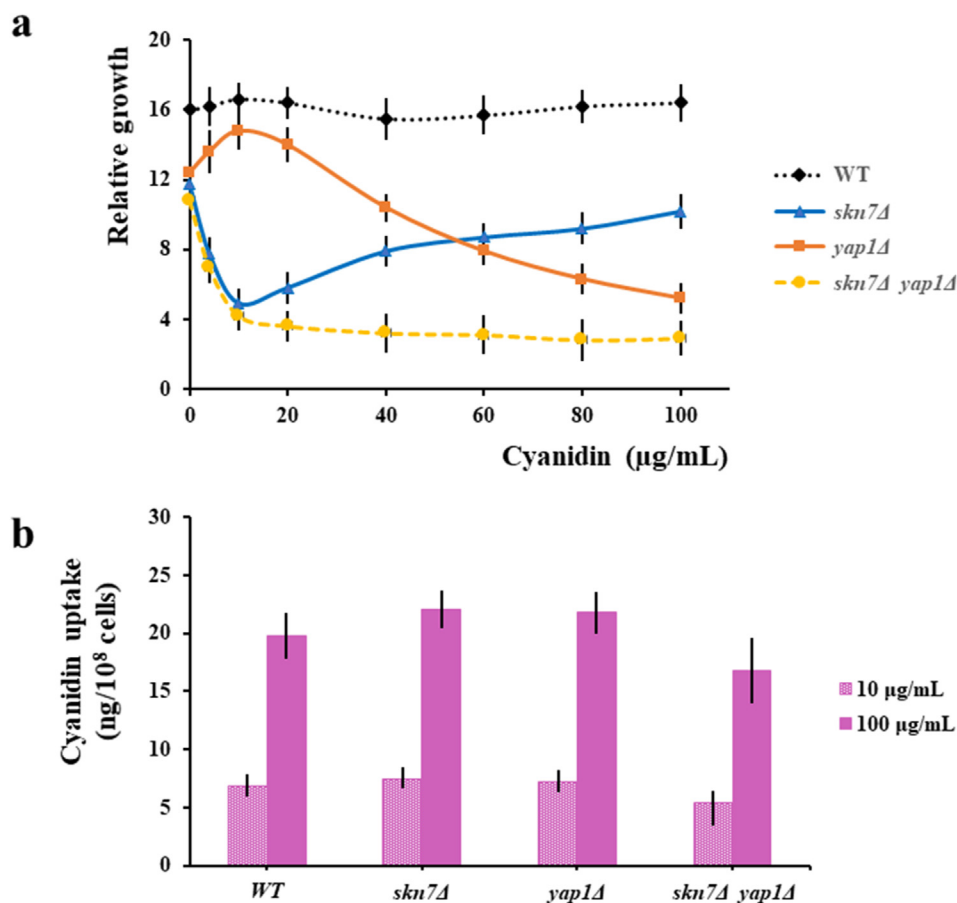


Figure 1. Effect of cyanidin on cell proliferation. (a) Relative growth at increasing cyanidin concentrations. Exponentially growing wild type (WT), *skn7Δ*, *yap1Δ* and double mutant *skn7Δ yap1Δ* cells were inoculated (5×10^5 cells/mL) in liquid SC supplemented with various amounts of cyanidin. Cell growth was determined spectrophotometrically at 600 nm (OD₆₀₀) after 16 h of cyanidin exposure. Relative growth was determined as the ratio OD_{600-16 h}/OD_{600-0 h} for each strain. (b) Cyanidin uptake. Exponentially growing cells were incubated for 2 h (30 °C, 200 rpm) with cyanidin (0, 10, or 100 μg/mL) before cyanidin uptake was determined as described in Materials and methods section. Values are mean ± SD of triplicate determination done on three biological repeats.

cyanidin impaired the growth of *skn7Δ* or *yap1Δ* knock-out cells (Figure 1a), albeit in a different manner. It was seen that the proliferation of *yap1Δ* was stimulated by low concentrations of cyanidin (5–20 $\mu\text{g}/\text{mL}$), but when increasing the dose, cell growth was perturbed, and concentrations higher than 20 $\mu\text{g}/\text{mL}$ diminished *yap1Δ* cell proliferation; *yap1Δ* growth was reduced to approximately half by exposure to 100 $\mu\text{g}/\text{mL}$ cyanidin (Figure 1a, red line). In contrast, the proliferation of the *skn7Δ* was impaired by low cyanidin concentrations (2–10 $\mu\text{g}/\text{mL}$), a trait that was no longer apparent at higher concentrations. In fact, at cyanidin concentrations higher than 20 $\mu\text{g}/\text{mL}$ the growth of *skn7Δ* improved, and at 80–100 $\mu\text{g}/\text{mL}$, the

proliferation of *skn7Δ* cells was not significantly different from that of non-exposed cells (Figure 1a, blue line). Deleting the *YAP1* gene in *skn7Δ* background prevented the growth recovery associated with increasing cyanidin concentration; in fact the growth of the double mutant *skn7Δ yap1Δ* was impaired by cyanidin at both low and high concentration (Figure 1a, yellow line). It was noted that the strains' different behavior to cyanidin exposure was not the result of different cyanidin uptake, as the WT, *skn7Δ* and *yap1Δ* cells accumulated similar amounts of cyanidin, at both low (10 $\mu\text{g}/\text{mL}$) and high (100 $\mu\text{g}/\text{mL}$) concentrations (Figure 1b). The cyanidin uptake recorded for

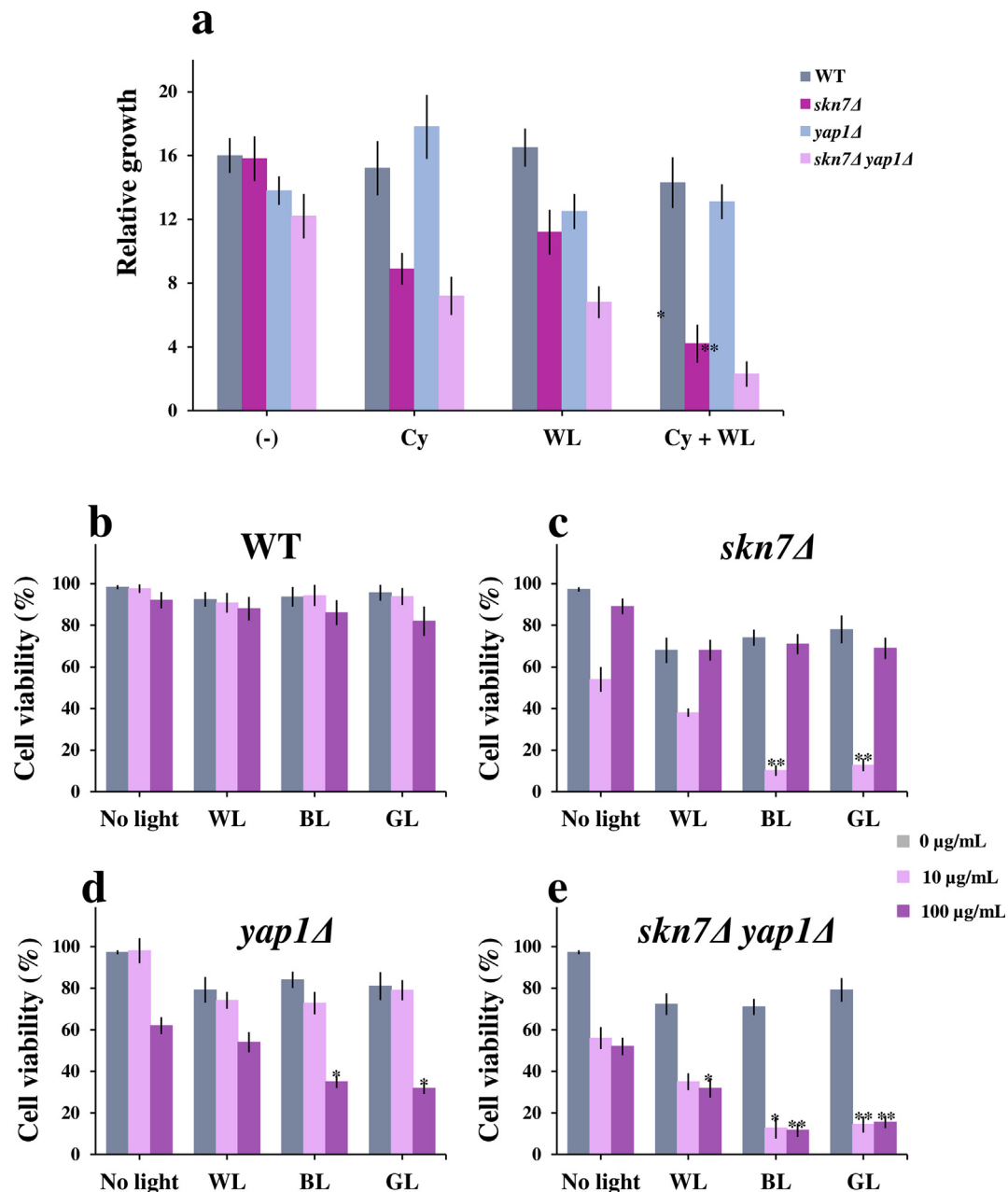


Figure 2. Effect of light exposure on cyanidin toxicity. (a) Cell proliferation. Exponentially growing wild type (WT), *skn7Δ*, *yap1Δ* and double mutant *skn7Δ yap1Δ* cells were inoculated (5×10^5 cells/mL) in a transparent multiwell plate containing SC supplemented or not with 10 $\mu\text{g}/\text{mL}$ cyanidin. To see the effect of illumination, plates were exposed to visible light for 2 h then incubated in the dark together with the control plates. Cell proliferation was determined spectrophotometrically at 600 nm (OD_{600}), 16 h after ceasing illumination. Relative growth was determined as the ratio $\text{OD}_{600-16 \text{ h}}/\text{OD}_{600-0 \text{ h}}$ for each strain. (b–e) Cell viability of cyanidin-treated cells exposed to light. Cells grown as in (a) were treated with the indicated concentrations of cyanidin then exposed to WL (white light, continuous spectrum), BL (blue light, $\lambda = 455 \text{ nm}$) or GL (green light, $\lambda = 527 \text{ nm}$) for 2 h as described in *Materials and methods* section. Cells were harvested and viability test was done by staining with methylene blue. Viability was examined for at least 300 cells from each of 3 biological repeats. Two-way ANOVA followed by Bonferroni's test, * $p < 0.05$, ** $p < 0.01$. Cy, cyanidin.

skn7Δ yap1Δ was slightly smaller, but this could be attributed to the poor growth of the double mutant under cyanidin exposure.

3.2. Visible light augments the sensitivity to cyanidin of cells lacking *Skn7*

While studying the effect of cyanidin on yeast growth it was accidentally noted that yeast cells spotted on cyanidin-containing agar plates proliferated slower if the plates were not taken to the dark incubator immediately after the spotting, but instead were kept under direct light for 1–2 h before being transferred to a dark environment (data not shown). It was previously shown that exposure to visible light is deleterious to both *skn7Δ* and *yap1Δ* cells, especially in the presence of photosensitizers, due to formation of singlet oxygen $^1\text{O}_2$ (Brombacher et al., 2006). To see if light alters the cyanidin toxicity, we inoculated yeast cells in SC medium containing 10 $\mu\text{g}/\text{mL}$ cyanidin and placed them under visible light for 2 h before shifting the plates to a dark incubator. It was noted that the combination between cyanidin and white light was deleterious to *skn7Δ* cells, which grew slower than WT, and even than *yap1Δ* cells (Figure 2a). Double knock-out mutant *skn7Δ yap1Δ* was even more sensitive to a combination of low cyaniding and light exposure (Figure 2a). Raising the cyanidin concentration (80–100 $\mu\text{g}/\text{mL}$) non-discriminately killed the yeast cells under illumination, as seen by the viability test, which indicated less than 15% viability for all strains (data not shown).

Light exposure clearly inhibited cell proliferation of *skn7Δ* and *skn7Δ yap1Δ* cells incubated with cyanidin (Figure 2a); we therefore tested the viability of the cells exposed to both light and cyanidin. It was noted that low cyanidin concentrations (10 $\mu\text{g}/\text{mL}$) significantly augmented the sensitivity of *skn7Δ* cells to white light (WL, continuous visible spectrum) but especially to monochromatic blue light (BL, $\lambda = 455 \text{ nm}$) and green light (GL, $\lambda = 527 \text{ nm}$) (Figure 2c, light purple bars). In contrast, 10 μM cyanidin did not significantly alter the viability of *yap1Δ* cells exposed to WL, BL or GL (Figure 2d, light purple bars) indicating that a functional *Skn7* is sufficient for cell survival under a combination of light exposure and low cyanidin.

It was surprising to note that increasing the cyanidin concentration improved the viability of *skn7Δ* cells exposed to WL, BL or GL (Figure 2c, dark purple bars), but not that of *yap1Δ* cells (Figure 2d, dark purple bars). This observation suggested that while a functional *Skn7* is sufficient to adapt to illumination under low cyanidin, Yap1 is needed for cell adaptation to high cyanidin under illumination. In this line of evidence, it was noted that the viability of double mutant *skn7Δ yap1Δ* was affected by illumination under both low and high cyanidin (Figure 2e).

3.3. High cyanidin stimulates Yap1 translocation to the nucleus through generation of ROS

The different behavior of *skn7Δ* and *yap1Δ* cells indicated that the two transcription factors may have complementary roles under cyanidin stress. That is, *Skn7* would be required for cell adaptation under low cyanidin, while Yap1 would be necessary for adaptation under high cyanidin. The observation that the *skn7Δ* growth was perturbed by low cyanidin, and that the *skn7Δ* cells gained tolerance when cyanidin concentration increased, suggested that cyanidin – when surpassing a threshold – may actually cause Yap1 activation for cell defense.

Yap1 is a key factor in regulating oxidative stress, such as exposure to H_2O_2 ; this is done primarily through Yap1 translocation from cytosol to nucleus, where it induces transcription of antioxidant genes (Delaunay et al., 2000). As some polyphenols were shown to induce Yap1 translocation to nucleus through generation of reactive oxygen species (ROS) (Maeta et al., 2007; Escoté et al., 2012) we wondered if cyanidin may also exert a pro-oxidant effect and cause Yap1 translocation to nucleus. To test this possibility, we used a GFP-tagged construct to monitor the cellular localization of Yap1 under cyanidin exposure. It was noted that under low cyanidin (10 $\mu\text{g}/\text{mL}$), Yap-GFP localized mainly in the cytosol, while under high cyanidin exposure (100 $\mu\text{g}/\text{mL}$), Yap-GFP concentrated in the nucleus, in both wild type and *skn7Δ* cells (Figure 3). This observation suggested that at high concentration cyanidin exerted a pro-oxidant effect, probably through generation of H_2O_2 (while at low concentrations cyanidin acts mainly as a photosensitizer). To detect whether exposure to high cyanidin generated H_2O_2 , we used cells transformed with a construct encoding peroxiredoxin-based roGFP2-Tsa2 Δ CR, a cytosolic redox sensor for H_2O_2 (Morgan et al., 2016). The peroxiredoxin-based H_2O_2 probes had been shown to be suitable for detecting variations in the cellular oxidative state as they are ~50% oxidized under ‘normal’ unstressed conditions and are equally responsive to increases and decreases in H_2O_2 (Morgan et al., 2016). It was noted that the level of oxidation of the redox-sensitive roGFP2-Tsa2 Δ CR (roGFP-Tsa2 Δ CR/OxD) was not significantly different in *skn7Δ* or *yap1Δ* compared to WT cells under normal growth conditions (Figure 4a-c, grey lines). In contrast, the level of roGFP-Tsa2 Δ CR/OxD was significantly higher in the double mutant *skn7Δ yap1Δ* even in the absence of cyanidin (Figure 4d, grey line), indicating that even under normal conditions, the level of ROS in *skn7Δ yap1Δ* cells was elevated. Exposure to low (10 $\mu\text{g}/\text{mL}$) cyanidin did not significantly alter the oxidative state of the cells in the first 30 min of exposure (Figure 4, light purple lines). In contrast, exposing yeast cells to high cyanidin (100 $\mu\text{g}/\text{mL}$) resulted in elevation of the roGFP-Tsa2 Δ CR/OxD in both WT and *skn7Δ* or *yap1Δ* cells (Figure 4a-c, dark

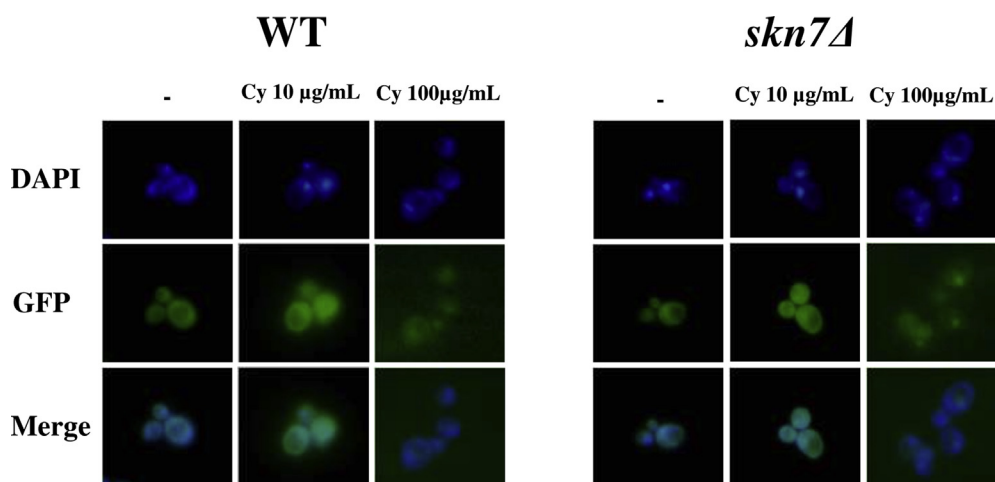


Figure 3. Effect of cyanidin on Yap1-GFP localization. Wild type (WT) and *skn7Δ* cells transformed with pRS425-Yap1-GFP were grown to exponentially-growing phase in SC-Leu before cyanidin was added to 10 $\mu\text{g}/\text{mL}$ or 100 $\mu\text{g}/\text{mL}$ (final concentration) and incubated for 2–4 h (30 °C, 200 rpm) before live cells were visualized by fluorescence microscopy using a GFP filter set, as described in *Materials and methods* section. Cy, cyanidin.

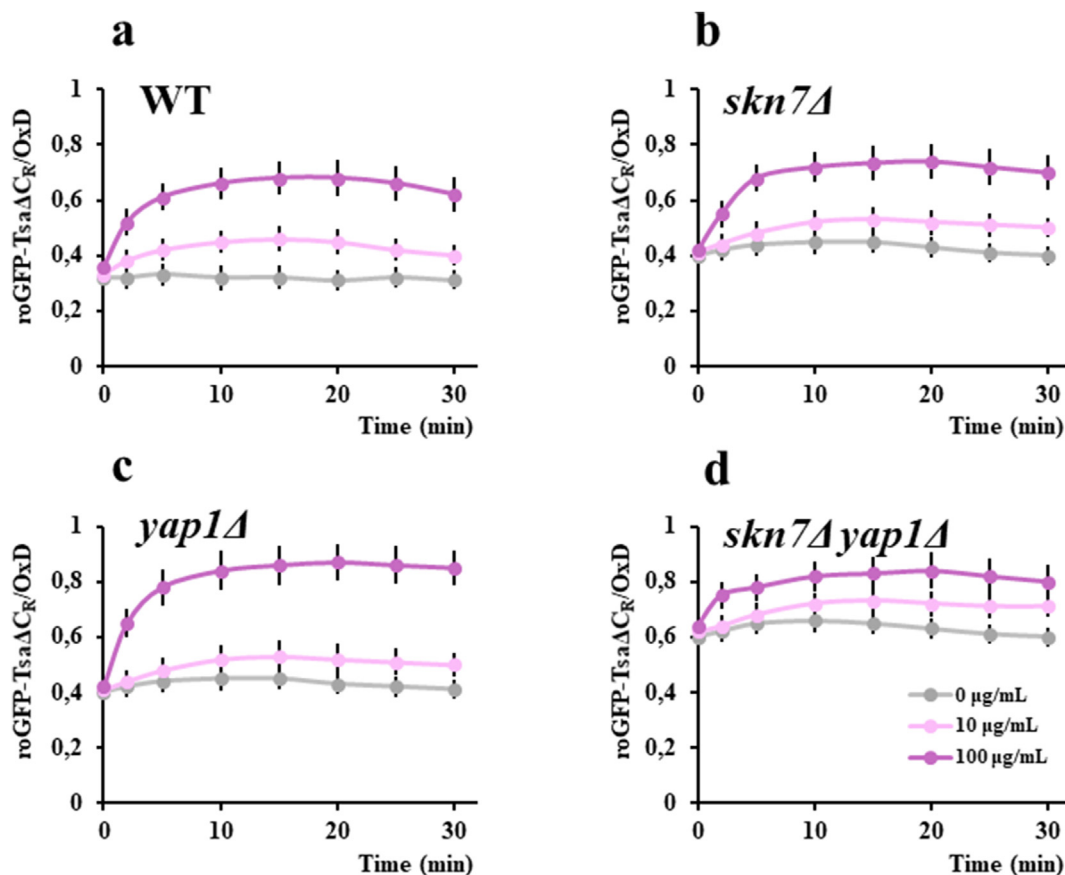


Figure 4. Oxidation of roGFP-Tsa2ΔCR by cyanidin. Late logarithmic growth-phase WT (a), *skn7Δ* (b), *yap1Δ* (c) and double mutant *skn7Δ yap1Δ* (d) cells transformed with p415-TEF-roGFP-Tsa2ΔCR (the plasmid harboring the construct for the H₂O₂-responsive redox sensor roGFP-Tsa2ΔCR) were treated with cyanidin (0, 10 and 100 μg/mL) directly in the multiwell plate used for the detection of sensor fluorescence, as described in *Materials and methods* section. The degree of sensor oxidation (OxD) is shown, with OxD = 1 for the fully oxidized probe and OxD = 0 for the fully reduced probe. Values are mean ± SD of triplicate determination done on three biological repeats.

purple lines); the elevation of roGFP-Tsa2ΔCR/OxD was significantly higher in *yap1Δ* cells (Figure 4c, dark purple line), indicative of a higher amount of ROS (H₂O₂) within the cells. The increase of roGFP-Tsa2ΔCR/OxD caused by cyaniding in *skn7Δ yap1Δ* cells was not significant (Figure 4d), probably due to high level of sensor oxidation under basal conditions.

4. Conclusions

Cyanidin was found to be deleterious to *S. cerevisiae* cells lacking either of the two main transcription factors which regulate the yeast response to oxidative stress in yeast, Skn7 and Yap1. The susceptibility of *skn7Δ* or *yap1Δ* to cyanidin was different though, suggesting that the roles of Skn7 and Yap1 in adaptation to cyanidin exposure are complementary: Skn7 is required for adaptation under low concentrations, while Yap1 is necessary for adaptation under high cyanidin. This conclusion was supported by the sensitivity manifested by the double mutant *skn7Δ yap1Δ* to both low and high cyanidin. The sensitivity of *skn7Δ* to low concentrations of cyanidin was augmented by cell exposure to visible light, indicating that cyanidin may act as a photosensitizer (Brombacher et al., 2006; Hessling et al., 2017; Galappaththia et al., 2018), raising awareness on the possible unwanted effects of utilizing anthocyanidin-rich supplements products associated with light exposure. At high concentrations, the cyanidin stress correlated with ROS generation, which in turn stimulated the cell response through Yap1 translocation to nucleus. Either way, it is clear that non-discriminatory consumption of supplements which are excessively enriched in

anthocyanidin-derived products need to be re-considered, or at least regarded with caution.

Declarations

Author contribution statement

Lavinia Liliana Ruta: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Eliza Oprea: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Claudia Valentina Popa: Performed the experiments; Analyzed and interpreted the data.

Ileana Cornelia Farcasanu: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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