A simple method for quantification of protochlorophyllide in etiolated Arabidopsis seedlings

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Abstract

Etiolated seedlings accumulate the chlorophyll biosynthesis intermediate protochlorophyllide (Pchlide) and measuring Pchlide can be important for characterising photomorphogenic mutants that may be affected in chloroplast development. In this chapter we outline a simple and sensitive method for quantifying Pchlide in extracts of Arabidopsis seedlings using fluorescence spectroscopy. This method can be easily adapted to study chloroplast development in a wide range of plant species.

Key words

Tetrapyrroles, Chlorophyll synthesis, Chloroplast development, Etioplasts, Fluorescence spectroscopy

Running Head

Protochlorophyllide qunantification in Arabidopsis

1 Introduction

1.1 The Importance of Measuring Pchlide in Photomorphogenic Research

This Chapter describes a simple and sensitive method to estimate protochlorophyllide (Pchlide) levels in Arabidopsis seedlings that can usefully be applied to a range of different photomorphogenic mutants. It is also suitable for use with most plant species with modification to the solvent volumes used. In dark-grown or etiolated seedlings of angiosperms the synthesis of the light-harvesting tetrapyrrole, chlorophyll is blocked at the biosynthetic intermediate Pchlide, which then accumulates ***(1)***. Figure 1 shows the Arabidopsis tetrapyrrole pathway. The block occurs because the enzyme Pchlide oxidoreductase (POR) in angiosperms requires light for activity ***(2)***. In lower plants and algae, a light-independent enzyme exists and Pchlide does not accumulate in darkness ***(1)***. In angiosperms the synthesized Pchlide is normally held bound to POR and NADPH in a complex that makes up a major component of the prolamellar body of etioplasts. Once a seedling is exposed to light Pchlide is rapidly converted to chlorophyllide (Chlide) and then esterified to provide chlorophyll for the new photosystems and POR is degraded resulting in the breakdown of the prolamellar body ***(3)***.

Photoreceptor regulation of chloroplast development is an important part of de-etiolation and photomorphogenesis ***(4)*** and measurement of Pchlide levels in dark-grown seedlings can be an extremely useful in the characterization of photomorphogenic mutants. The understanding of two classes of photomorphogenic mutants in particular has benefited from the ability to measure Pchlide levels in etiolated seedlings. Firstly, mutants that have a constitutively photomorphogenic phenotype, that is respond in the dark as if they are in the light ***(5)***, frequently show elevated levels of Pchlide if the signalling components have a role

in chloroplast development. Examples include the *cop1* mutant ***(6)***, the *pif1* ***(7)*** and *pif3* ***(8)*** mutants, and the *pifQ* mutant lacking PIF1, PIF3, PIF4 and PIF5 ***(9)***. See Figure 2 for an illustration of the higher Pchlide levels observed in the *pifQ* mutant. Various hormone response pathways also regulate Pchide levels via these components, including, for example, gibberellins ***(10)*** and ethylene ***(11)***. In all cases, the signalling pathways result in an increase in Pchlide via induction of *HEMA1* expression and increased activity of glutamyl tRNA reductase (GluTR), the rate limiting step for all tetrapyrrole synthesis ***(8, 12)***. In this type of analysis the measurement of Pchlide acts as a proxy for a role in chloroplast development without the added complications of interpreting the effect of white light on chlorophyll accumulation. Under conditions that result in the accumulation of Pchlide, seedlings photobleach on transfer to white light. This can be seen for example in the *flu* mutant, which lacks a repressor of GluTR activity, resulting in accumulation of Pchlide in the dark (see Figure 2) and photobleaching in the light ***(13)***. The effect of elevated Pchlide is exacerbated under conditions in which POR levels are reduced, for example by far-red light that inhibits expression of *POR* genes, but does not permit Pchlide photoconversion ***(14)***. Photobleaching on transfer to white light is the result of the production of singlet oxygen generated by the reaction of excited Pchlide with molecular oxygen. This results in inhibition of nuclear-encoded photosynthesis gene expression ***(15)*** and can ultimately lead to cell death ***(16)***. The production of singlet oxygen from elevated Pchlide levels has also been demonstrated in *pif* mutants ***(17)***.

The second class of photomorphogenic mutants for which Pchlide measurements have proved useful are the phytochrome chromophore-deficient mutants ***(18)***. These mutants lack heme oxygenase and phytochromobilin synthase activities (see Figure 1) resulting on long hypocotyl phenotypes due to phytochrome deficiency. This class of mutants includes *elongated hypocotyl 1* (*hy1*) and *hy2* ***(19-21)***. Analysis of Pchlide levels in the equivalent

tomato mutants *yg-2* and *au* showed that they also contained reduced levels of Pchlide ***(22)*** supporting a model in which heme feeds back to inhibit the plant tetrapyrrole pathway ***(23)***. Reduced Pchlide has also been demonstrated for chromophore-deficient mutants of pea and Arabidopsis ***(24)*** as shown in Figure 2 for *hy1*.

1.2 Approaches to measuring Pchlide

There are a range of different methods that can be used to measure Pchlide. Most of these require extraction from seedlings by solvents, but it is also possible to measure Pchlide in situ. Low-temperature (77 K) fluorescence spectroscopy has been used to identify four different fluorescent Pchlide forms corresponding to different molecular organizations of the POR proteins binding the Pchlide ***(25)***. This technique has been applied to analysis of both constitutively photomorphogenic mutants ***(26)*** and phytochrome chromophore-deficient mutants ***(27)***. More frequently, Pchlide is measured following solvent extraction, where again a wide range of methods can be used for analysis. Some laboratories use high performance liquid chromatography (HPLC) where the highly absorbent and fluorescent properties of chlorophyll precursors make detection relatively easy ***(28-30)***. Pchlide has also been successfully analysed by mass spectrometry following separation by HPLC ***(31, 32)***. This method makes it easier to distinguish the different forms of Pchlide (see later).

For routine measurement of Pchlide, spectroscopic methods are far more straightforward. For reliable quantification, absorption spectroscopy is the best method as the Pchlide concentration can be calculated using its molar absorption coefficient in acetone of 31,100 M^{-1} cm^{-1} at 626 nm ***(33)***. This method was used for the analysis of tomato seedlings ***(22)***, but the amount of tissue needed is not ideal for routine analysis in Arabidopsis seedlings. Instead, it is possible to take advantage of the highly fluorescent properties of chlorophyll

precursors and estimate Pchlide amounts using fluorescence spectroscopy. It should be noted that fluorescence does not have units and all measurements are of relative fluorescence enabling only a comparison with other genotypes or treatments in the experiment. Fluorescence can also be an unreliable measure of the amount of a compound because it can be subject to fluorescence quenching in which photons emitted from the fluorescent molecules are absorbed by other molecules in the sample and not detected by the spectrophotometer. This will result in lower values at higher concentrations of Pchlide. Nevertheless, the small amounts of Arabidopsis tissue required and the ease and reliability of the measurements makes this a very useful, routine assay for characterising the effect of light signalling mutants on chloroplast development.

2 Materials

1. Growth media: 1% (w/v) agar with 0.5 x MS salts.
2. Extraction solvent: Acetone:0.1M NH_4OH (90/10; v/v) should be prepared in advance and kept at 4°C prior to use (see **Note 1**).
3. 1.5 mL microcentrifuge tubes and polypropylene pestles.
4. Chilled bench top centrifuge (see **Note 2**).
5. 1 mL fluorescence glass cuvettes.
6. Fluorescence spectrophotometer.

3 Methods

3.1 Pchlide extraction

Arabidopsis seedlings should be grown in complete darkness to ensure there is no conversion of Pchlide to Chlide. There are a variety of standard methods for doing this, but typically seedlings are grown on Petri dishes containing 1% (w/v) agar with 0.5 x MS media ***(34)***. Germination is promoted by 2-3 d at 4°C followed by 2 h white light (~100 µmol.m^{-2}.s^{-1}) before transfer to the dark. 5 d dark growth at 22°C should be sufficient for maximum Pchlide accumulation. Even when grown in a dark growth cabinet it is recommended that Petri dishes are wrapped twice in foil (or placed in a tin). Harvesting should take place in a dark room under very low levels of green safelight as the POR enzyme is not activated at these wavelengths. The extraction method used is based on the method of Rebeiz et al ***(35)***.

1. Excise 20-30 seedlings (count exactly how many) by cutting near the base of the hypocotyl and transfer to a labelled microcentrifuge tube kept on ice (see **Note 3**).
2. Add 0.4 mL ice cold acetone: 0.1M NH_4OH (9/1; v/v).
3. Homogenise tissue using a polypropylene pestle.
4. Centrifuge for 5 min in a chilled bench top centrifuge (see **Note 2**) at top speed (> 12,000 x *g* is recommended).
5. Transfer the supernatant into a new, labelled microcentrifuge tube.
6. Add a further 0.4 mL ice cold acetone: 0.1M NH_4OH (9/1; v/v) to the first tube with the pellet and repeat the extraction.
7. Repeat centrifugation step.

8. Combine supernatants from the two extraction steps (steps 5-7). This is your Pchlide sample for analysis (see **Note 4**).
9. Put samples on ice, cover in foil and bring out of the dark room (see **Note 5**).

3.2 Pchlide measurement

1. Set the baseline of the fluorescence spectrophotometer to zero using 80% (v/v) acetone.
2. Measure samples at the peak value of ~636 nm (see **Notes 6-8**) following excitation at 440 nm. As there is some variation in background fluorescence between samples this can be normalised by also recording emission at 750 nm. Pchlide does not emit at this wavelength.
3. Calculate Pchlide as relative fluorescence on a per seedling basis, or if seedling morphology is different, on a per mg fresh weight basis.

Notes

1. The solvent solution is stable over a long period but, as with all solvents, do not pipette directly from the bottle to avoid plastic contamination.

2. If a chilled benchtop centrifuge is unavailable then a room temperature one can be used. In this case, keep samples chilled before and after centrifugation.

3. A lot more Pchlide is present in the cotyledons than in the hypocotyls and a measurement per seedling will mostly reflect cotyledon Pchlide. If the morphology of the seedlings being

tested is quite different then it can be best practice to also weigh seedlings so that can be presented on a per seedling and per gram fresh weight basis.

4. The Pchlide extracted using acetone: 0.1M NH_4OH (9/1; v/v) is a mixture of Pchlide and esterified Pchlide, or protochlorophyll (Pchl). The Pchl, which is generally in lower amounts, can be removed by washing with an equal volume of hexane ***(35)***, but where examined there was little observed difference between relative amounts of Pchlide and Pchl ***(22)***. For this reason, a hexane wash is not recommended for Pchlide estimation in Arabidopsis given the small volumes involved.

5. Once Pchlide is in solution it does not have to be kept in safelight conditions. As with all pigments keep cold and covered where possible to reduce light-mediated degradation, which will happen over time.

6. When measuring fluorescence always record the peak height not the height at a single wavelength (e.g. 636 nm). Contamination of a sample can shift the position of the peak, but has less effect on peak height. In addition, as discussed earlier, sample readings can be inaccurate due to fluorescence quenching. It is important to be measuring Pchlide when fluorescence increases linearly with sample concentration. The solvent volumes and tissue amounts suggested here should ensure this is the case, but if in doubt this can be determined empirically using dilutions of the most concentrated samples.

7. If the seedlings have been exposed to light some of the Pchlide will have been converted to Chlide. This will be seen as a peak (or shoulder) at ~670 nm. If this is absent then your samples are of good quality.

8. There are actually numerous form of Pchlide in plants and the fluorescent measurements recorded will be the sum of these. In etiolated seedlings photoactive Pchlide is generally in the form of 3,8-divinyl Pchlide *a* ***(36)***. Monovinyl Pchlide *a* is also commonly detected ***(28)***, but Pchlide *b,* which differs in having an aldehyde group instead of a methyl group at the C7 position, has only been detected in green plants and not in etiolated seedlings *e.g*. ***(30)***.

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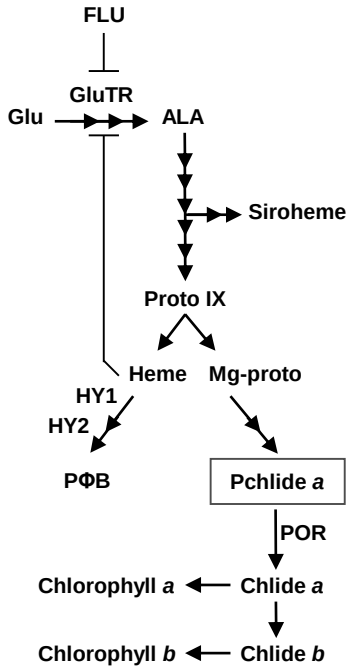
Figure legends

Fig. 1 The tetrapyrrole pathway in Arabidopsis. ALA, 5-aminolevulinic acid, Chlide, chlorophyllide; FLU, repressor of GluTR; Glu, glutamate; GluTR, glutamyl tRNA reductase encoded by *HEMA* genes; HY1, heme oxygenase 1; HY2, phytochromobilin synthase; Mg-proto, magnesium protoporphyrin IX; POR, protochlorophyllide oxidoreductase; Pchlide, protochorophyllide; Proto IX, protoporphyrin IX

Fig. 2 Measurement of protochlorophyllide (Pchlide) by fluorescence spectroscopy. Fluorescence spectra taken following excitation at 440 nm in wild-type seedlings and three mutants, *flu* (the *fluorescent* mutant lacking a repressor of GluTR), *pifQ* (comprised of *pif1, pif3, pif4 and pif5*) and *hy1* (deficient in heme oxygenase 1). See text for further information on these mutants. Inset, quantification of relative fluorescence (RFU) at the ~636 nm peak from three independent biological repeats. Data shown are mean ± standard error.



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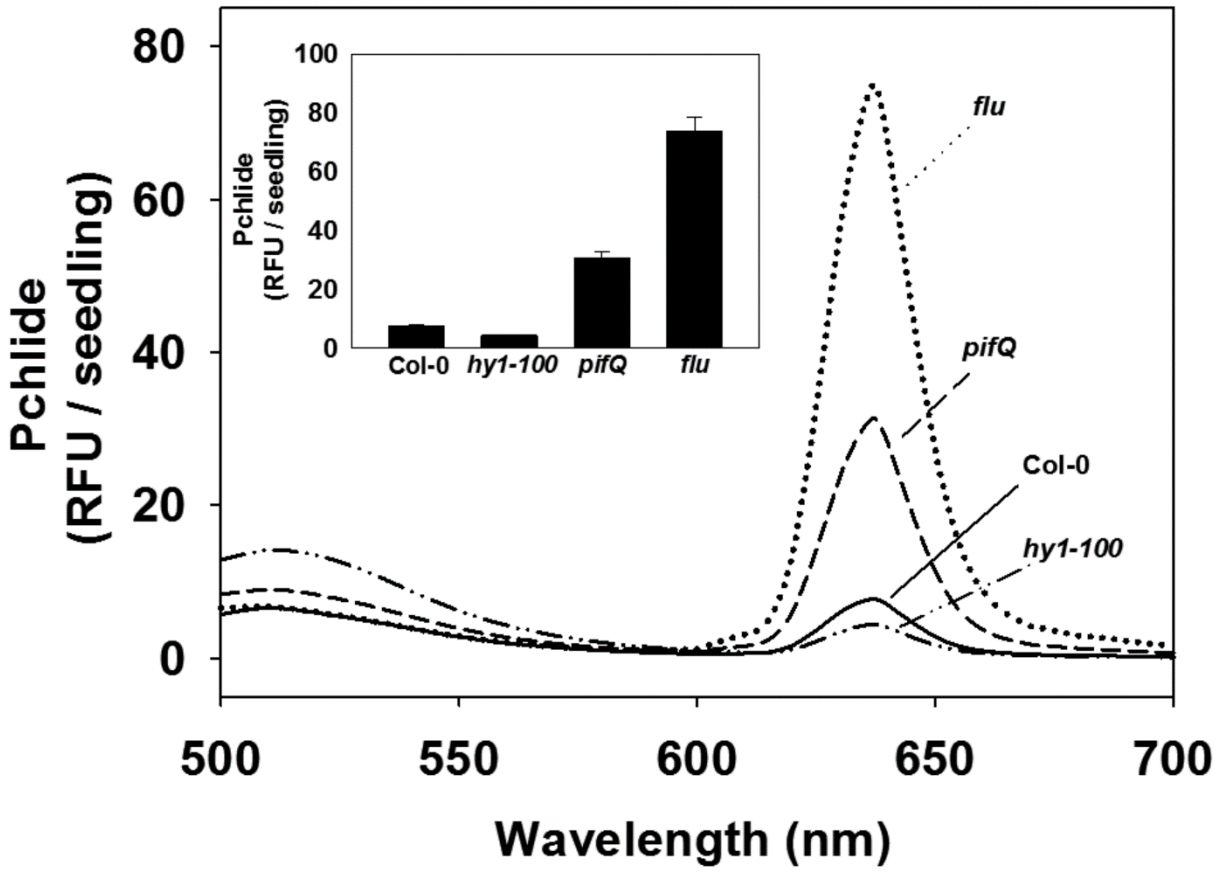

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