CHAPTER TWELVE

In vitro assay and inhibition of 9-*cis*-epoxycarotenoid dioxygenase (NCED) from *Solanum lycopersicum* and *Zea mays*

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Abstract

The article reports methods for the expression and assay of 9-*cis*-epoxycarotenoid cleavage dioxygenase (NCED), an enzyme involved in the biosynthesis of phytohormone abscisic acid in plants. A method for the preparation of the unstable substrate 9′-*cis*-neoxanthin from fresh spinach is described. The inhibition of *Solanum lycopersicum* NCED by a series of aryl hydroxamic acid inhibitors is illustrated, and inhibitors D2 and D4 are assayed against NCED isozymes from *Zea mays*.

1. Introduction

Carotenoids are C_{40} polyene terpenoids biosynthesised by phototrophic organisms such as plants, algae and certain bacteria (Walter & Strack, 2011). Carotenoids are important metabolites across many different species. Mammals must obtain carotenoids from their diet, and approximately 40–50 carotenoids are consumed in the average human diet. Some carotenoids, such as β-carotene or pro-vitamin A carotenoids, are metabolized, whilst others are absorbed intact (Khachik, 2006; Maiani et al., 2009). In plants, carotenoids and their derivatives (termed apocarotenoids) are important hormones. Abscisic acid and strigolactone are derived from the carotenoids 9′-*cis*-neoxanthin and β-carotene respectively. Abscisic acid is involved in a number of different plant processes including seed dormancy and plant response to water deficit (Iuchi et al., 2001; Taylor, Sonneveld, Bugg, & Thompson, 2005). Similarly, strigolactone has been implicated in processes such as rhizosphere signalling and shoot branching (Akiyama, Matsuzaki, & Hayashi, 2005; Gomez-Roldan et al., 2008; Umehara et al., 2008). More recently, β-cyclocitral, derived from the cleavage of β-carotene, has been shown to regulate root architecture in monocots and eudicots (Dickinson et al., 2019; Felemban, Braguy, Zurbriggen, & Al-Babili, 2019).

The biosynthesis of apocarotenoids from carotenoids is in part controlled by a family of enzymes termed carotenoid cleavage dioxygenases (CCDs). CCDs catalyse the oxidative cleavage of the polyene backbone of carotenoids into two apocarotenoid products (Harrison & Bugg, 2014). In the case of abscisic acid biosynthesis, the enzyme 9-*cis*-epoxycarotenoid cleavage dioxygenase (NCED) catalyzes the selective oxidative cleavage of the 11′,12′ double bond in 9′-*cis*-neoxanthin or the 11,12 double bond in 9-*cis*-violaxanthin to give a C_{25} aldehyde product and a second aldehyde called xanthoxin (North et al., 2007; Taylor et al., 2005) (Fig. 1). Xanthoxin is then further metabolized by the enzymes xanthoxin dehydrogenase (ABA2) and abscisic-aldehyde oxidase (AAO3) to produce

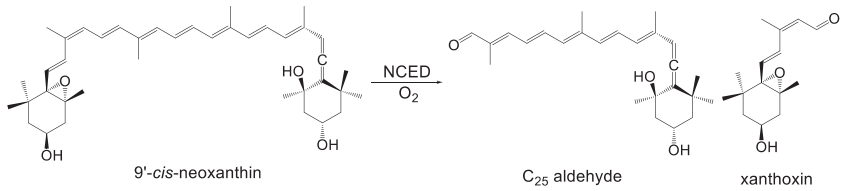


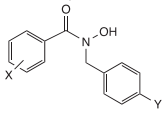
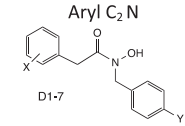
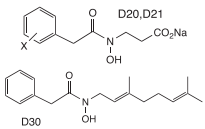
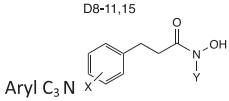
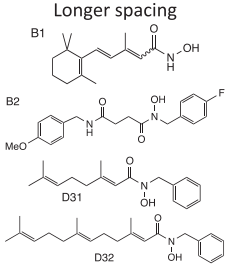
Fig. 1 Schematic representation of the NCED catalyzed 11′,12′ cleavage of 9′-*cis*-neoxanthin to xanthoxin and C_{25} aldehyde.

abscisic acid (Cheng et al., 2002; González-Guzmán et al., 2002; Seo et al., 2000). NCED is the rate limiting step in the biosynthesis of ABA, and NCEDs are selective for the 9-*cis* and 9′-*cis* isomers of violaxanthin and neoxanthin, respectively, showing no activity against all-*trans* carotenoid substrates (Schwartz, Tan, McCarty, Welch, & Zeevaart, 2003).

The enzyme 9-*cis*-epoxycarotenoid cleavage dioxygenase 1 (NCED1, encoded by *viviparous 14*, *vp14*) from *Zea mays* (maize) was the first biochemically characterized carotenoid cleavage enzyme (Schwartz, Tan, Gage, Zeevaart, & McCarty, 1997). Homologs of NCED have been found in a wide variety of different plant species such as *Arabidopsis thaliana* (thale cress) and *Solanum lycopersicum* (tomato) (Burbidge, Grieve, Jackson, Thompson, & Taylor, 1997; Tan et al., 2003). There are varying numbers of NCED homologs in different species. For example, in *S. lycopersicum* there are only two NCEDs. However, in *Z. mays* and *A. thaliana* there are five different NCED homologs, at least one of which responds sensitively to increased ABA accumulation during cellular water deficit, while others have roles in seed dormancy or have unknown physiological roles (Frey et al., 2012; Lee, Lee, & Seo, 2015; Lefebvre et al., 2006; Tan et al., 2003).

In order to explore the function of CCD enzymes and apocarotenoids in plants, there have been several approaches to develop selective CCD inhibitors. To date, a number of different inhibitors targeting NCED (and other CCDs) have been developed (Table 1). The first reported inhibitor of NCED was abamine, derived from nordihydroguaiaretic acid (Creelman, Bell, & Mullet, 1992; Han et al., 2004). *In vitro*, abamine inhibits cowpea (*Vigna unguiculata*) NCED by ~40% (~40% reduction in activity compared to the control) at a concentration of 100 μM (K_i 38.8 μM), and in vivo abamine causes a number of phenotypic effects including inhibiting stomatal closure and ABA accumulation under osmotic stress. A subsequent study resulted in the development of abamineSG, which inhibits cowpea NCED ~80% at 100 μM (K_i 18.5 μM) (Kitahata et al., 2006). Moreover, AbamineSG inhibited ABA

Table 1 Inhibition of SlNCED1 by aryl hydroxamic acid inhibitors.[a]

Inhibitor class	Inhibitor	*X*	*Y*	% Inhibition @ 100 μM
Aryl C_1 N 	F1	4-OMe	H	0
	F2	4-OMe	F	0
	F3	3,4-$(OMe)_2$	H	2
	F4	3,4-$(OMe)_2$	F	0
	F5	3-Cl	H	5
	F6	3-NH_2	H	7
Aryl C_2 N 	D1	4-OH	H	27
	D2	4-OH	F	29
	D3	3,4-$(OH)_2$	F	4
	D4	4-OMe	F	33
	D5	3,4-$(OMe)_2$	H	8
	D6	3,4-$(OMe)_2$	F	18
	D7	3,4-OCH_2O	F	33
	D20	4-OMe		22
	D21	4-naphthyl		39
	D30			42
D8-11,15 	D8	3,4-$(OMe)_2$	CH_2Ph	40
	D9	4-OMe	CH_2Ph	27
	D10	3,4-$(OMe)_2$	*n*-octyl	14
	D11	4-OMe	*n*-octyl	15
	D12	3,4-$(OMe)_2$	H	11
	D13	4-OMe	H	13
	D15	4-OMe	CH_2PhF	24
Longer spacing 	B1			5
	B2			13
	D31			10
	D32			10

[a]Inhibition data for compounds F1–4, D1–13, B1 reported in Sergeant et al. (2009); inhibition data for other compounds not previously reported. Chemical synthesis and characterization data for hydroxamic acids reported as follows: F1–4, D1–13, B1, Sergeant et al. (2009); F5, F6, Sergeant et al. (2013); D15, Van Norman et al. (2014); D20–21, D30–32, B2, Harrison et al. (2015).

accumulation in plants under osmotic stress by 77% at 100 μM. Both abamine and abamineSG showed less than 20% inhibition at 100 μM against CCD1 and CCD7 in vitro (Kitahata et al., 2006). A series of sesquiterpene-like compounds have also been shown to inhibit NCED, albeit less effectively than abamine(SG) (Boyd et al., 2009). The most effective of these compounds inhibited *Arabidopsis* NCED3 with a K_i of 57 μM, and when tested in vivo, one compound was shown to lower levels of ABA in osmotically stressed plants, and slightly promoted germination at 0.1 μM but slightly inhibited germination at concentrations above 0.33 μM.

A series of hydroxamic acid based compounds have also been shown to be effective inhibitors of NCED and CCD1 (Sergeant et al., 2009). Hydroxamic acids are metal chelators which are potent inhibitors of a number of different metal dependent enzymes. Variation in the length of the carbon chain between the aryl group (proximal to the carbonyl of the hydroxamic acid) and the nitrogen of the hydroxamic acid (the aryl-*N* length, Table 1) was hypothesised to change the specificity of the hydroxamic acids against different CCD cleavage reactions. Compounds targeted against 9,10 cleavages, aryl-C_2N, showed >95% inhibition at 100 μM against tomato (*S. lycopersicum*) CCD1a but also showed moderate activity against tomato NCED at 100 μM. Aryl-C_3N compounds which were designed to target 11,12/11′,12′ cleavages showed moderate activity against tomato CCD1a at 100 μM but also included the most effective compound against tomato NCED1 (40% by compound D8) (in this study abamine showed only 20% inhibition against tomato NCED) at 100 μM.

In planta, a number of phenotypic effects have been seen on application of hydroxamic acid CCD inhibitors (Sergeant et al., 2009). In *Arabidopsis*, hydroxamic acid inhibitors were shown to advance the timing of bud outgrowth by between 1 and 3 days. Similarly, whole plants treated with hydroxamic acids exhibited a bushy phenotype and increased shoot branching. When compound D6 is applied to wild type *A. thaliana*, there is a twofold increase in the number of shoot branches produced (Sergeant et al., 2009). Mediation of the shoot branching phenotype is thought to occur through inhibition of CCD7 and/or CCD8, which controls the biosynthesis of strigolactone, a phytohormone which inhibits shoot branching in plants (Gomez-Roldan et al., 2008; Umehara et al., 2008).

More recently, two hydroxamic acid compounds (D4 and D7) were shown to promote germination in osmotically-stressed tomato seeds and to prevent ABA-mediated inhibition of germination in lettuce (Awan et al., 2017). Effects on seed germination are believed to be mediated through inhibition of NCED. NCED is the rate limiting step in the biosynthesis of ABA, a germination

inhibitor that is widely implicated in seed dormancy induction and maintenance (Finch-Savage & Leubner-Metzger, 2006). Additionally, with the hydroxamic acids F1 and F2, phytotoxic effects on *A. thaliana* have been observed which can be observed through bleaching of the plants (Sergeant et al., 2013). This phytotoxicity effect has been shown to occur partially through inhibition of metalloenzyme *p*-hydroxy-phenylpyruvate dioxygenase, a known herbicide target (Sergeant et al., 2013), however, studies indicate that a second, unknown enzyme, is also involved. One hydroxamic acid, D15 has also been shown to affect lateral root branching in *A. thaliana* (Van Norman et al., 2014). The biochemical target of this inhibitor has been identified as β-cyclocitral and constitutes a new role for carotenoids *in planta* (Dickinson et al., 2019).

In vitro assays of NCED are challenging and low throughput, due to an unstable substrate, and an unstable enzyme. This report will provide practical details for 9′-*cis*-neoxanthin substrate preparation, NCED expression and assay, and inhibition of NCED by a larger collection of hydroxamic acids than previously published, and testing of hydroxamic acids D2 and D4 against NCED isozymes from *Z. mays*.

2. 9-*cis*-neoxanthin isolation from spinach and characterisation by ultraviolet–visible spectroscopy (UV-Vis) and high-performance liquid chromatography (HPLC)

2.1 Equipment

- Sieve (≈1 mm pore).
- Buchner funnel with Whatman grade 1 filter paper.
- Hand blender or pestle and mortar.
- 1 L separating funnel.
- Glass chromatography column.
- Rotary evaporator.
- UV-Vis spectrometer.

2.2 Buffers and reagents

- Spinach.[1]
- Methanol.
- Sodium bicarbonate.

[1] We observed that a lower yield of 9′-*cis*-neoxanthin was obtained from spinach stored at 4 °C for >1 day.

- Butylated hydroxytoluene.
- Saturated sodium chloride solution (brine).
- Diethyl ether.
- Alumina resin.
- Petroleum ether.
- Ethanol.
- Triton X-100.

2.3 Procedure

1. To cold methanol (200 mL) add fresh organic spinach (≈100 g), sodium bicarbonate (0.4 g) and butylated hydroxytoluene (0.04 g).
2. Homogenize the spinach with a hand blender or pestle and mortar for five minutes.
3. Filter the resulting solution through a sieve (≈2 mm pore) to give a dark green liquid.
4. Filter the dark green liquid under suction using a Buchner funnel.
5. Partition the filtrate between cold saturated sodium chloride (100 mL) and cold diethyl ether (200 mL) using a large separating funnel.
6. Remove and retain the ether layer and again wash the organic layer with cold diethyl ether (200 mL).
7. Combine the ether extracts and wash with cold saturated sodium chloride solution (100 mL).
8. Concentrate the ether fraction under reduced pressure to give a dark green oil.
9. Activate alumina (10 g) by the addition of distilled water (1.0 mL) and petroleum ether (20 mL) and allow to stir for 5 min at room temperature.
10. Prepare a column under gravity with the activated alumina.
11. Wash the column with petroleum ether (50 mL).
12. Resuspend the spinach resin in diethyl ether (2 mL) and petroleum ether (10 mL) and apply to the column.
13. Apply 1: 1 petroleum ether: diethyl ether (100 mL) followed by diethyl ether (200 mL) to the column and discard the flow through.
14. Finally elute the 9-*cis*-neoxanthin with 5% ethanol in diethyl ether (100 mL).
15. Add triton X-100 to a final concentration of 0.1% (v/v).
16. Remove the solvent under reduced pressure to give a yellow solid.
17. Resuspended in ethanol (1 mL) and store in the dark at −20 °C.
18. Characterize by UV-Vis spectroscopy or HPLC (see Section 4.3 for HPLC method).

19. Data: λ_{max} 416, 440 and 467 nm; High-Resolution Mass Spectrometry (HRMS) (MicroTOF) 601.4251, calc. 601.4244 for $C_{40}H_{56}O_4$. ε at 440 nm: 1.33×10^5 M^{-1} cm^{-1}.

2.4 Notes and results

Chemically, 9′-*cis*-neoxanthin is unstable and susceptible to light, pH, temperature and oxygen. This sensitivity is due to the presence of functional groups including an epoxide, an alkyne and conjugated double bonds within its structure. Whilst commercial preparations of 9′-*cis*-neoxanthin are available, due to its inherent instability sometimes fresh preparations are preferable.[2] 9′-*cis*-neoxanthin can be prepared freshly from spinach (*Spinacia oleracea*) leaves, which have a high natural abundance of neoxanthin (Tonucci et al., 1995) via published protocols (Sergeant et al., 2009).[3]

Characterisation of 9′-*cis*-neoxanthin is possible by thin layer chromatography (Schwartz et al., 1997), however, in our hands we found this technique inconclusive, probably due to its instability. However, 9′-*cis*-neoxanthin can be identified via its characteristic UV-Vis spectrum, which has been extensively reported in the literature (Britton, 1985; Cholnoky et al., 1969).[4] 9′-*cis*-neoxanthin has a distinctive 3 peak spectrum, with absorbance maxima at 467, 439 and 416 nm[5] (Fig. 2A). In our preparations in ethanol solution, we observed absorbance maxima of 465, 439 and 417 nm, in good agreement with the literature and spectra of commercial 9′-*cis*-neoxanthin (Table 2). Isolation of 9′-*cis*-neoxanthin can also be confirmed by mass spectrometry, with an m/z of 603.4244 Da (Fig. 2B).

3. Overexpression of *S. lycopersicum* and *Z. mays* NCED in *Escherichia coli*

3.1 Equipment

- Cell disruptor or sonicator.
- Floor standing centrifuge with rotor.
- Bench top centrifuge with rotor for 50 mL tubes.
- Empty gravity column or FPLC system

[2] Neoxanthin should always be stored at −80 °C.

[3] We found organic spinach gave the highest yields of 9′-*cis*-neoxanthin.

[4] Due to the highly conjugated nature of carotenoids, they likely exist in a number of different isomeric forms.

[5] Variations in the local environment (solvent, temperature) and *cis-trans* isomerisation of alkenes on the polyene backbone will likely lead to slight variations in the absorbance maxima.

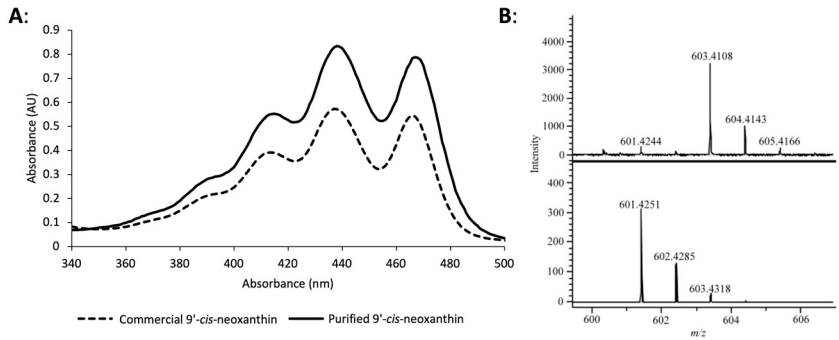


Fig. 2 (A) UV-Visible absorbance spectrum of purified and commercial 9′-*cis*-neoxanthin in ethanol. The absorbance maxima are listed in Table 2. (B) HRMS of purified 9′-*cis*-neoxanthin from spinach.

Table 2 Absorbance maxima recorded for purified 9′-*cis*-neoxanthin (in ethanol) compared with literature values for all-*trans*-neoxanthin and 9′-*cis*-neoxanthin (Britton, 1985, Cholnoky et al., 1969).

	All-*trans*-neoxanthin (literature)	9′-*cis*-neoxanthin (literature)	Purified 9′-*cis*-neoxanthin	Commercial 9′-*cis*-neoxanthin (CaroteNature)
Absorbance maxima (nm)	467	467	465	466
	440	439	439	437
	415	416	417	414

3.2 Buffers and reagents

- Resuspension buffer (phosphate buffered saline (PBS)) pH 7.4, 10% glycerol (v/v), 0.05% Triton X-100 (v/v) and 1 mM 2-mercaptoethanol.
- Wash buffer (PBS pH 7.4, 10% glycerol (v/v), 0.05% Triton X-100 (v/v), and 1 mM 2-mercaptoethanol).
- Elution buffer (50 mM) trisaminomethane-HCl (Tris-HCl) pH 8.0, 20 mM reduced glutathione, 10% glycerol (v/v), 1 mM 2-mercaptoethanol and 0.05% Triton X-100 (v/v).
- Lysozyme.
- 1,4-dithiothreitol.
- DNAse I
- 1, 10 phenanthroline
- Glutathione-*S*-transferase (GST) resin (loose resin or prepacked column).

3.3 Procedure

1. Transform competent *E. coli* BL21 pRosetta cells with a plasmid containing cloned NCED using standard protocols.
2. Inoculate 10 mL of lysogeny broth (LB) media containing the appropriate antibiotic with a single colony of *E. coli*. Incubate overnight at 37 °C, 180 rpm.
3. Inoculate 500 mL of LB media containing appropriate antibiotics with 10 mL of the overnight culture. Incubate at 37 °C, 180 rpm until the optical density (OD) at 600 nm reaches 0.6 absorbance units (AU).
4. Add isopropyl β-d-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Incubate overnight at 20 °C, 180 rpm.
5. Harvest the cells at 4220 × *g* at 4 °C for 15 min and either freeze at −80 °C or used as follows.
6. Resuspend the cell pellet into 10 mL resuspension buffer.
7. Add lysozyme and 1,4-dithiothreitol to a final concentration of 25 μg/mL and 5 mM respectively.
8. Incubate the resuspended cells at room temperature for 20 min.
9. Lyse the cells by cell disruption at 20.1 kpsi or sonication at 50 Hz for 5 min.
10. Add DNAse I to a final concentration of 5 μg/mL and incubate on ice for 20 min.
11. Centrifuge at 20,300 × *g* at 4 °C for 15 min before filtering the supernatant through a 0.2 μm filter.
12. For assays involving cell free extract, the cell free extract can be used without any further purification.
13. Add 1,10-phenanthroline to a final concentration of 1 mM to the cell free extract and incubate on ice for 15 min.
14. Add the cell free extract to GST resin prepared according to the manufacturer's instructions.
15. Pass the cell free extract through the column a total of three times.
16. Wash the column with 25 mL of wash buffer.
17. Elute into GST elution buffer (10 mL) plus 20 mM reduced glutathione, 10% (v/v) glycerol (v/v), 1 mM 2-mercaptoethanol (BME) and 0.05% Triton X-100 (v/v).
18. Concentrate the protein with a 50 kDa centrifugal filter unit.
19. Buffer exchange wash buffer using a PD10 gel filtration column used according to the manufacturer's instructions.
20. Concentrate further using a 10 kDa centrifugal filter.
21. Aliquot the protein, snap freeze in liquid nitrogen and store at −80 °C.

3.4 Notes and results

For our in vitro assays, NCED from tomato (*S. lycopersicum*) and *Z. mays* were cloned into a vector with an *N*-terminal glutathione-*S*-transferase (GST) tag. GST tags are commonly used over other tags (for example His_6 tags) for the expression of NCED since metal affinity tags can cause issues with metal dependent enzymes (Iuchi et al., 2001).[6] In addition, the large soluble nature of the GST-tag can aid the expression and solubility of proteins. This characteristic quality is particularly useful for CCDs, which frequently associate with the membrane and can cause aggregation.

For this work, we cloned and over expressed 5 isoforms of *Z. mays* NCED (VP14) (*ZmNCED1* (Zm00001eb050380)*, 2, 3A, 3B* and *9*) and one isoform of *S. lycopersicum* NCED (*SlNCED1*, Solyc07g056570 (previously known as *LeNCED*)). Mature NCED proteins lacking the chloroplast transit peptide (cTP) exhibit more activity than immature NCEDs retaining the cTP (Qin & Zeevaart, 1999). Maize *NCED*s, as identified in Vallabhaneni et al., were amplified from the coding sequence adjacent to the predicted *N*-terminus cTP coding sequence using the primers in Table 3 (Vallabhaneni, Bradbury, & Wurtzel, 2010). A full-length cDNA homologous to the truncated *ZmNCED2* (NM_001154055) was used to design primers. Primers contained 5′ extensions with *Eco*RI and *Bam*HI restriction sites in the forward and reverse primers, respectively.

Using SlNCED1, we tested a number of different expression strains including BL21, pRosetta BL21, Origami and pRosetta gami. From our expression trials we observed the highest levels of expression of SlNCED1 in BL21 pRosetta.[7] For *Zm*NCED1, 2, 3A and 3B expression was observed in *E. coli* BL21. However, no expression was observed for *Zm*NCED9 in either *E. coli* BL21 or BL21 pRosetta.

NCEDs can also be readily purified using the GST affinity tag, although final yields may be variable depending upon the instability of the enzyme (see notes and discussion for enzyme assays). The purification protocol is standard for soluble proteins, but we do suggest the addition of protease inhibitors and a reducing agent (such as tris (2-carboxyethyl) phosphine hydrochloride (TCEP), beta-mercaptoethanol (BME) or dithiothreitol (DTT)). We also add a small amount of Triton X-100 to aid with the solubility of NCED.

[6] We have expressed and purified other CCD enzymes (*A. thaliana* CCD7 and CCD8) in our laboratory using an N-terminal His_6 tag (Harrison et al., 2015).

[7] Our *Z. mays* and *S. lycopersicum* NCEDs were not sequence optimized for recombinant expression in *E. coli*.

Table 3 Primers used to amplify *ZmNCED* fragments for insertion into pGEX-4T-1 and expression as a GST-fusion protein without chloroplast transit peptide.

Target, cTP length (AA)	Direction	Primer sequence[a]
ZmNCED1, (23)	F	**ATATGGATCC**GCCTCCAATTCCGTCAGGTTCTCGCC
	R	**ATCATGAATTC**TCAGGCCGCCTGGGCCTCGAG
ZmNCED2, (65)	F	**ATTAGGATCC**GCAGCGGCAGCGGCCAACTCG
	R	**AGTCGGAATTC**TCAGGCCTGGGCCTCTAGCTC
ZmNCED3a, (46)	F	**ATTAGGATCC**GCCGCCGCTCCCAAGTGGAACCCG
	R	**ACGTAGAATTC**CTAGGCCTGCCGCTGCAGCTC
ZmNCED3b, (52)	F	**ATCTGGATCC**GCGGCCGCCAAGTGGAACCC
	R	**ATCATGAATTC**CTAGGCCTGCCGCTGCAGCTC
ZmNCED9, (23)	F	**ACGTGGATCC**GCCTCCAACTCCGTCAGGTTC
	R	**ACTGGAATTC**TCAGGCCTGGGCCTCGAGCTC

[a]5′ extension indicated in bold and *Bam*H1 and *Eco*RI restriction sites are underlined.

4. NCED assay and enzyme inhibition assays

4.1 Equipment

- HPLC system
- C18 HPLC column (Phenomenex HyperClone) C_{18} reverse phase HPLC column (5 μm BDS, 130 Å, 250 ×4.6 mm)
- 5 mL glass vials
- Compressed nitrogen gas line.

4.2 Buffers and reagents

- Iron (II) sulfate
- Sodium ascorbate
- Ethyl acetate
- Cotton wool filters
- Methanol
- Acetonitrile

4.3 Procedure

1. For assays with purified NCED, defrost on ice or use fresh. Add iron (II) sulfate and sodium ascorbate to a final concentration of 1 mM and incubate on ice for 10 min.
2. For assays involving cell free extract, add 1.3 mM iron (II) sulfate and 1.3 mM sodium ascorbate. Incubate on ice for 5 min before addition to the assay.
3. To NCED assay buffer (130 or 150 μL) add fresh 9′-*cis*-neoxanthin extract (25 μL, ≈80 μM), cell lysate containing overexpressed NCED enzyme (50 μL) or purified NCED (20 μL, ≈50 μg).
4. Incubate in the dark (using black Eppendorf tubes) at room temperature for 15 min.
5. Add water (700 μL) and transfer to glass vials.
6. Extract into ethyl acetate (3 × 700 μL).
7. Filter the ethyl acetate through cotton wool and dry under a stream of nitrogen.
8. Dissolve the residue in methanol (300 μL).
9. Inject 50 μL into a C18 column maintained at room temperature at a flow rate of 0.5 mL/min.
10. Elute with 95:5 acetonitrile:methanol, shifting to 85:10:5 acetonitrile:methanol:ethyl acetate over 20 min. Over the next 5 min, shift the gradient to 95:5 acetonitrile:methanol and hold for an additional 20 min.
11. C_{25} product and unreacted 9′-*cis*-neoxanthin are observed at 440 nm.
12. Retention times: 9′-*cis*-neoxanthin, 10.2 min; C_{25} product, 6.5 min.
13. For assays involving inhibitors, the inhibitors were dissolved in either neat ethanol or neat DMSO, and a total of 2 μL of inhibitor stock was added to the assay to give a final concentration of 100 μM, ensuring no more than 5% (v/v) DMSO or ethanol in the final reaction mixture.

4.4 Notes and results

NCED assays were conducted in a total volume of 200 μL in black 1.5 mL tubes to minimize non-specific *cis–trans* isomerisation of polyene substrates and products. Due to abundance of protein in the cell free extract, and the absence of any enzymes within K12 *E. coli* (we expressed in BL21(DE3) *E. coli* cells) with competing activity, we performed the majority of NCED inhibition assays using *E. coli* cell extracts containing overexpressed NCED. However, NCED can be purified using the GST-tag. Interestingly, we observed 40-fold higher specific activity of NCED in crude *E. coli* extracts

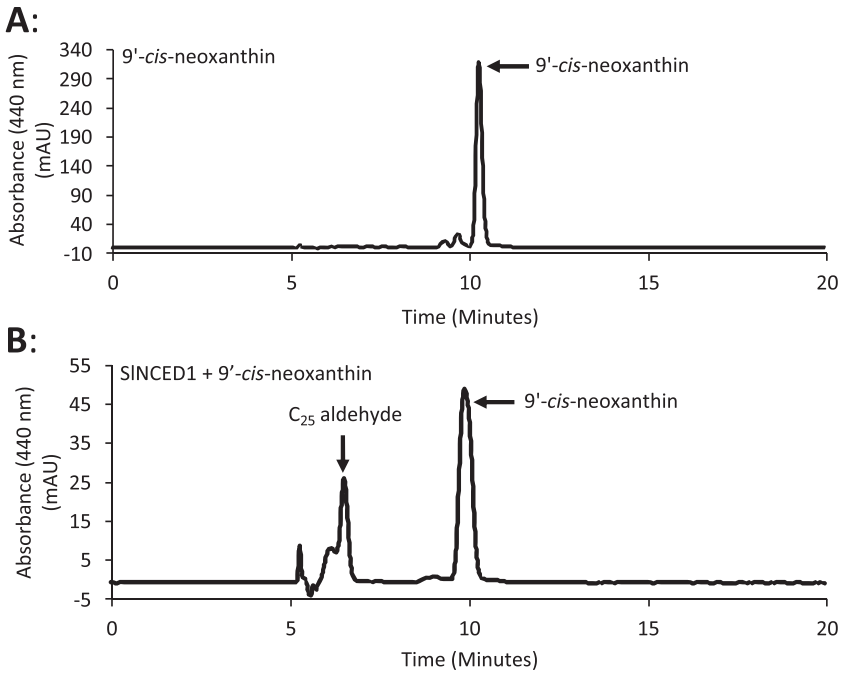


Fig. 3 HPLC chromatogram of *Le*NCE1D catalyzed cleavage of 9′-*cis*-neoxanthin (A, top) and 9′-*cis*-neoxanthin (B, bottom). C_{25} aldehyde peak can be seen eluting at 6.5 min in the chromatogram of the enzyme catalyzed reaction.

(180 nmol/h/mg protein) compared with the purified enzyme reported in Schwartz et al. (2003) (3.5 nmol/h/mg protein) (Schwartz et al., 2003). This different may be due to inherent instability of NCED, which would become apparent during purification.[8]

Given that cell free extract containing overproduced SlNCED1 was used for the assays, it was not possible to determine the exact concentration of SlNCED1 present in the assays. However, we estimate that approximately 100 μg of protein was added to each assay. We also observed that a 2:1 (v/v) excess of cell lysate (from 3 mL of cell free extract prepared from a 500 mL culture of *E. coli*) containing overproduced GST-SlNCED1 to 9′-*cis*-neoxanthin was required to allow the reaction to proceed to 50% completion. We estimate that each assay contained approximately 60 μM of 9′-*cis*-neoxanthin (purified from spinach).

[8] We have observed that purification of some NCEDs can lead to loss of activity.

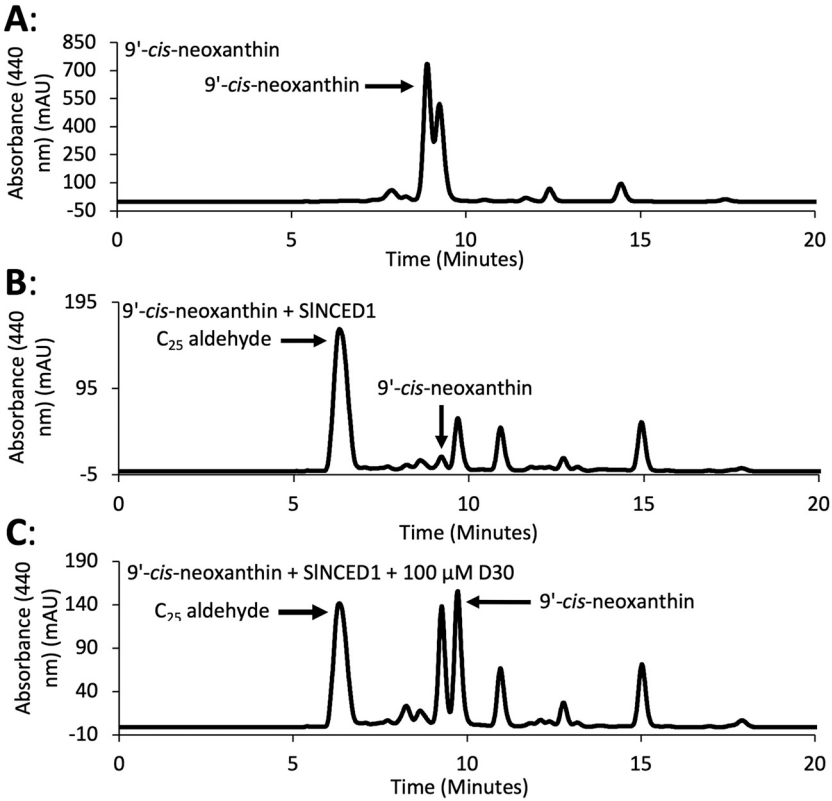


Fig. 4 (A) HPLC chromatogram of 9′-*cis*-neoxanthin control. (B) SlNCED1 catalyzed cleavage of 9′-*cis*-neoxanthin (~71% conversion). (C) SlNCED1 catalyzed cleavage of 9′-*cis*-neoxanthin in the presence of 100 µM D30 (~50% conversion).

Upon incubation of SlNCED1 with 9′-*cis*-neoxanthin for 20 min at 25 °C, a product peak at ~6.5 min was observed at 440 nm, corresponding to that reported by Sergeant et al. (2009) (Fig. 3A and B). Integration of the product and substrate peaks observed on the HPLC chromatogram was used to follow the course of the reaction. For assays with inhibitors, inhibitors were added at a final concentration of 100 µM.[9] Percentage inhibition was calculated by working out the percentage conversion for each reaction from integration of the substrate and the product peaks for each assay at 440 nm (Fig. 4A, B and C).

[9] Inhibitors were solubilized in either DMSO or ethanol.

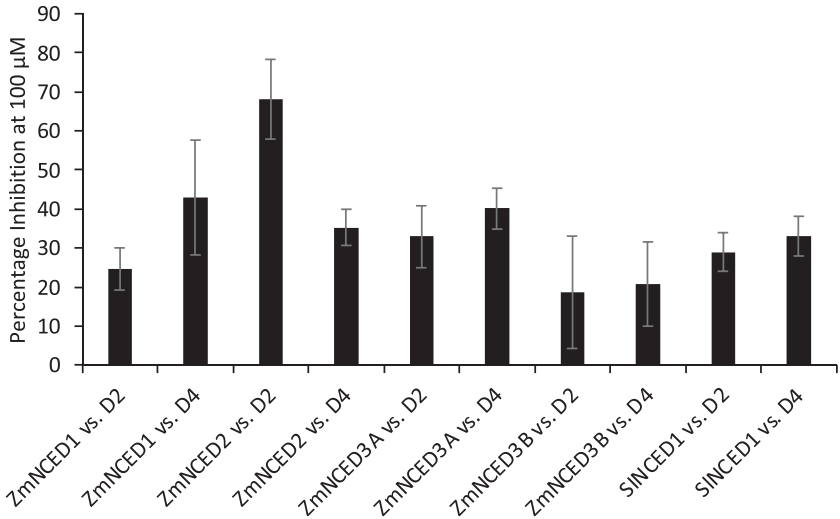


Fig. 5 Inhibition shown by hydroxamic acid inhibitors D2 and D4 against *Z. mays* NCED1, NCED2, NCED3A, NCED3B, NCED9 and *S. lycopersicum* NCED1.

Inhibition data for some hydroxamic acid inhibitors has been published elsewhere and is summarized here (Table 1) (Awan et al., 2017; Sergeant et al., 2009, 2013; Van Norman et al., 2014). The synthesis of all hydroxamic acid inhibitors tested against SlNCED1 have been reported (Awan et al., 2017; Harrison et al., 2015; Sergeant et al., 2009, 2013; Van Norman et al., 2014). Of the novel compounds we have screened here against SlNCED1 (F5, F6, D20, D21, D30, D31 and D32), the highest level of inhibition was seen with compound D30 (42% at 100 μM), closely followed by compound D21 (39% at 100 μM) and D20 (22% at 100 μM). Neither of the F-series inhibitors were effective against SlNCED1 (5% and 7% for F5 and F6 at 100 μM respectively), nor were compounds D31 or D32 (both 10% at 100 μM).

We screened the hydroxamic acid inhibitors D2 and D4 for activity against homologs of *Zm*NCED. As mentioned, we were unable to express *Zm*NCED9. However, against *Zm*NCED1, 2, 3A and 3B we observed varying levels of effectiveness of D2 and D4 (Fig. 5). We did not observe complete inhibition for any of the *Zm*NCED homologs against D2 or D4. The highest level of inhibition we observed was 68.0% ± 10.2% for inhibitor D2 against *Zm*NCED2. Against *Zm*NCED3A, D2 and D4 performed comparably (32.9% ± 8.0% for D2 and 40.2% ± 5.2% for D4). Similarly with *Zm*NCED3B, no significant difference was observed between D2 and

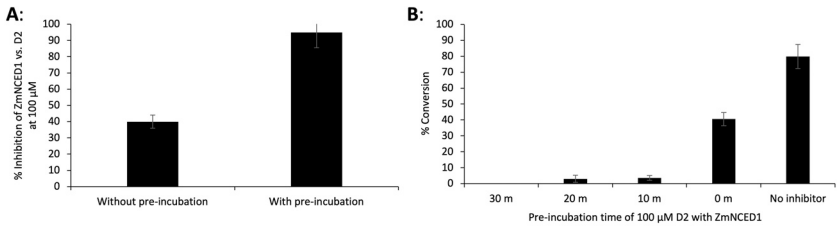


Fig. 6 (A) Percentage inhibition observed at 100 µM D2 verses cell free extract containing over produced *Zm*NCED1 with and without pre-incubation of D2. (B) Percentage conversion of 9′-*cis*-neoxanthin to C_{25} aldehyde by *Zm*NCED1 following pre-incubation of *Zm*NCED1 with compound D2 for various lengths of time.

D4 (18.7% ± 14.4% for D2 and 20.8% ± 10.7% for D4). A larger difference was seen for *Zm*NCED1 (24.6% ± 5.4% for D2 and 42.9% ± 14.7% for D4), but the largest difference was observed for *Zm*NCED2 (68.0% ± 10.2% for D2 and 35.3% ± 4.5% for D2). With the exception of D2 verses *Zm*NCED2, there was little difference in the levels of inhibition seen for D2 and D4 against SlNCED1 (29% and 33% respectively).

In addition, we also observed time dependent inhibition effect against *Zm*NCED1 with inhibitor D2. In this instance, a higher level of inhibition was obtained when the inhibitor D2 was pre-incubated. In samples that were pre-incubated, almost 100% inhibition of the enzyme was observed (Fig. 6A). However, in samples without pre-incubation, inhibition levels of less than 40% were seen (Fig. 6A). To discount the effect of other factors within the *E. coli* cell free extract, we purified *Z. mays* NCED1 via GST affinity chromatography. *Z. mays* NCED1 was shown to effectively turn over 9′-*cis*-neoxanthin to xanthoxin and the C_{25} aldehyde. However, when D2 was pre-incubated with the enzyme for different lengths of time (either 30, 20 or 10 min), no turnover was observed (Fig. 6B). Partial inhibition was seen when D2 was added simultaneously with the substrate.

5. Discussion

We have shown here an efficient and optimized protocol for the over-expression, purification and assay of NCED, and the purification of its substrate 9′-*cis*-neoxanthion from spinach. Purified 9′-*cis*-neoxanthin can be characterized by its distinct UV-Vis absorption profile as well as by high resolution mass spectrometry.

NCEDs from tomato and maize can be readily expressed and purified in *E. coli*. We performed our inhibition assays using cell free extract containing over expressed NCED. This method was partially due to the fact that NCEDs (in particular SlNCED1) can be unstable. One particular concern with performing assays with cell free extract is non-specific binding of the inhibitor molecules to other soluble proteins within the cell free extract. However, we have used cell free extract containing other overexpressed CCDs (*Le*CCD1) and have observed inhibition of greater than 95% for several inhibitors.

The strongest inhibition of *S. lycopersicum* NCED is observed by aryl C_2-N inhibitors in the D series. This finding is consistent with the positioning of the hydroxamic acid at a greater distance from the first aromatic ring, as proposed in Sergeant et al. (2009). The shorter aryl C_1-N analogues in the F-series inhibit LeCCD1, which cleaves carotenoid substrates via a 9,10-cleavage reaction, but show little of no inhibition of NCED, which cleavages via an 11,12-cleavage reaction (Sergeant et al., 2009). Compared to the compounds reported in Sergeant et al. (2009), somewhat higher inhibition was observed using compound D30, containing a terpene-like amino-substituent. Compounds with an aryl C_3-N spacing also inhibit NCED, with a variable structure-activity profile but slightly lower levels of inhibition than the aryl C_2-N series. Examination of the crystal structure of *Z. mays* VP14, a closely related NCED enzyme which shares significant sequence homology with SlNCED1, indicates that the active site and substrate binding tunnel are highly hydrophobic (as to be expected given the nature of the carotenoid substrates) (Messing et al., 2010). This analysis provides a possible explanation as to why compounds which can better exploit this hydrophobicity (for example the aryl-C_2N and -C_3N compounds) are better inhibitors (in comparison to aryl C_1N compounds).

We have reported shoot branching phenotypes, linked to inhibition of strigolactone biosynthesis, with compounds D2, D4, D5 and D6 showing the clearest shoot branching phenotype in *A. thaliana* (Sergeant et al., 2009). We have also reported seed germination phenotypes, linked to inhibition of abscisic acid biosynthesis (Awan et al., 2017) *in planta* for several of these hydroxamic acid compounds. Compounds D4 and D7 show the clearest seed germination phenotype in tomato, indicating that this phenotype is controlled by NCED inhibition (Awan et al., 2017). In further support of this finding, D4 was found to reduce ABA content in tomato seeds overexpressing *SlNCED1*, promote germination in tomato and tobacco overexpressing *SlNCED1* and lettuce sown at high temperatures (Awan et al., 2017).

Since many plant species have multiple NCED homologs, and to explore whether homologs are differentially inhibited, we cloned and expressed five different NCED homologs from *Z. mays*. Using cell free extracts containing overexpressed *Zm*NCED, the inhibitors D2 and D4 were assayed at 100 μM concentrations. By comparing the inhibition profiles of D2 and D4 versus NCED1, 2, 3 A and 3B both inhibitors show 20%–40% inhibition, apart from D2 verses *Zm*NCED2, which shows approximately 65% inhibition. However, if one particular isozyme was responsible for an observed phenotype when conducting an experiment at 1 mM concentration *in planta*, then one might expect greater than 90% inhibition for that enzyme at 100 μM of inhibitor in vitro. There may well be additional targets for these inhibitors, which could lead to a discrepancy between in vitro and *in planta* observations. Not only is there a very high sequence similarity between the five NCEDs in *Z. mays*, some hydroxamic acids (D12 and D13) have been shown to inhibit other iron dependent dioxygenases such as the *E. coli* catechol dioxygenase MhpB and HPPD (Sergeant et al., 2013). As such, the hydroxamic acids could be inhibiting another non-heme oxygenase enzymes.

We observed that pre-incubation of inhibitors with cell free extract containing overexpressed *Zm*NCED1 resulted in different levels of inhibition than was observed without pre-incubation. In samples that were pre-incubated, almost 100% inhibition of the enzyme was observed. However, in samples without pre-incubation inhibition levels of less than 40% were seen. With purified *Zm*NCED1, when D2 was pre-incubated with the enzyme for different lengths of time, either 30, 20 or 10 min, no turnover was observed. Partial inhibition was seen when D2 was added simultaneously with the substrate. This time dependent inhibition effect has been seen with hydroxamic inhibitors of *p*-hydroxy-phenylpyruvate dioxygenase (HPPD) (Sergeant et al., 2013). In this case inhibitors were shown to inhibit HPPD to a greater extent on pre-incubation of the inhibitor with the enzyme. This effect was speculated to be due to oxidation of the Fe(II)-HA complex to Fe(III)-HA (where HA represents the hydroxamic acid). Fe(III) would bind the hydroxamic acid more strongly, thus resulting in the increased levels of inhibition observed (Sergeant et al., 2013).

6. Conclusions

Seed germination, induction of seed dormancy by various environmental conditions, and plant response to cellular water deficit are controlled by the plant signalling hormone abscisic acid, the biosynthesis of which is

controlled by NCED (Taylor et al., 2005). However, within *Arabidopsis*, *Z. mays* and many other plant species, there are multiple NCED homologs, and it is unknown which specific NCED enzyme is the cause of observed phenotypes. NCED-specific inhibitors could therefore be useful for understanding gene function or for practical applications to control plant traits. We therefore investigated whether there were any differences in enzyme inhibition between different NCED isozymes in *Z. mays*, by expressing *Z. mays* NCED1, NCED2, NCED3A, NCED3B, and NCED9. *Zm*NCED9 expressed poorly in *E. coli*, but we were able to assay against the other NCED isozymes. Inhibition of all NCED isozymes by compounds D2 and D4 was observed, but with somewhat higher levels of inhibition against NCED2.

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In vitro assay and inhibition of 9-cis-epoxycarotenoid dioxygenase (NCED) from Solanum lycopersicum and Zea mays

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