RESEARCH ARTICLE

Barley Viridis-k links an evolutionarily conserved C-type ferredoxin to chlorophyll biosynthesis

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Short title: Barley Viridis-k Encodes a C-type Ferredoxin

One-sentence summary: The aerobic Mg-protoporphyrin IX monomethyl ester cyclase of the chlorophyll biosynthetic pathway obtains electrons from a ferredoxin with a C-terminal extension.

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ABSTRACT

Ferredoxins are single-electron carrier proteins involved in various cellular reactions. In chloroplasts, the most abundant ferredoxin accepts electrons from photosystem I and shuttles electrons via ferredoxin NADP+ oxidoreductase to generate NADPH or directly to ferredoxin dependent enzymes. In addition, plants contain other isoforms of ferredoxins. Two of these, named FdC1 and FdC2 in Arabidopsis thaliana, have C-terminal extensions and functions that are poorly understood. Here we identified disruption of the orthologous FdC2 gene in barley (Hordeum vulgare L.) mutants at the Viridis-k locus; these mutants are deficient in the aerobic cyclase reaction of chlorophyll biosynthesis. The Mg-protoporphyrin IX monomethyl ester cyclase is one of the least characterized enzymes of the chlorophyll biosynthetic pathway and its electron donor has long been sought. Agroinfiltrations showed that the viridis-k phenotype could be complemented in vivo by Viridis-k but not by canonical ferredoxin. VirK could drive the cyclase reaction in vitro and analysis of cyclase mutants showed that in vivo accumulation of VirK is dependent on cyclase enzyme levels. The chlorophyll deficient phenotype of viridis-k mutants suggests that VirK plays an essential role in chlorophyll biosynthesis that cannot be replaced by other ferredoxins, thus assigning a specific function to this isoform of C-type ferredoxins.

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INTRODUCTION

The chlorophyll molecule is a magnesium-containing tetrapyrrole synthesized in several consecutive steps each catalyzed by a specific enzyme (Tanaka and Tanaka, 2007; Bryant et al., 2020). One of the least understood steps is the conversion of Mg-protoporphyrin IX monomethyl ester (MPE) to protochlorophyllide, which is a six-electron oxidation. In this reaction the fifth ring E is formed which is unique to chlorophylls. The reaction is catalyzed by an MPE cyclase, adding a carbonyl group to C13\(^1\) as well as forming a carbon-carbon bond between C13\(^2\) and the bridge carbon between rings C and D (Figure 1). There are two unrelated enzymes that perform this reaction. One is an anaerobic enzyme where the C13\(^1\) carbonyl group comes from water and the other is an oxygen dependent enzyme where the carbonyl group instead comes from molecular oxygen (Walker et al., 1989; Porra et al., 1995; Wiesselmann et al., 2020). It is the latter enzyme, from here on just the cyclase, which is found in plants (Tottey et al., 2003; Chen et al., 2017) and is of special interest to the present study. This enzyme is a carboxylate bridged diiron monooxygenase and contains a characteristic iron binding motif EX\(_n\)EXXHX\(_n\)EX,EXXH where two iron atoms are bound by the glutamates and histidines (Berthold and Stenmark, 2003).

This type of enzyme requires a reductant and the plant cyclase is reduced with electrons from ferredoxin (Stuart et al., 2020) (Figure 1).

Plants and algae contain multiple chloroplast ferredoxins that are iron-sulfur proteins of the Fe\(_2\)S\(_2\) type and catalyze single electron transfer reactions. The iron-sulfur cluster is coordinated by cysteines in a CX\(_4\)CX\(_2\)CX\(_n\)C motif. The pattern of chloroplast ferredoxin expression, in different parts and under different conditions, suggests that they play important functions in various metabolic pathways since they are central electron donors in chloroplast metabolism (Terauchi et al., 2009; Hanke and Mulo, 2013). The classical ferredoxin participates in photosynthesis and passes electrons to ferredoxin NADP\(^+\) oxidoreductase (FNR), which in turn reduces NADP\(^+\) to NADPH (Tagawa and Arnon, 1962). However, ferredoxins can directly donate electrons to ferredoxin dependent enzymes involved in processes such as sulfur and nitrogen assimilation, tetrapyrrole metabolism and fatty acid biosynthesis to name a few (Hanke and Mulo, 2013). Plants contain four major types of conserved ferredoxins (Hanke et al., 2004; Voss et al., 2011). The leaf-type is primarily expressed in photosynthetic tissues and accepts
electrons from photosystem I as a part of photosynthetic electron transport while root-type is expressed mainly in non-photosynthetic tissues and is tuned for accepting electrons from NADPH via FNR in order to support ferredoxin dependent enzymes (Shinohara et al., 2017). In addition to these canonical ferredoxins, plants contain two ferredoxins with an extended C-terminus and are thus referred to as FdC1 and FdC2 in Arabidopsis thaliana (Voss et al., 2011). Their functions are not well established. The FdC1 isoform has been shown to accept electrons from photosystem I or NADPH via FNR but does not seem to be able to reduce NADP+ via FNR (Voss et al., 2011; Guan et al., 2018). Similarly, FdC2 can also be reduced by photosystem I or NADPH via FNR and may be found in redox dependent stromal ribonucleoprotein complexes (Kolton et al., 2011).

An in vitro cyclase assay utilizing recombinant proteins was recently developed (Stuart et al., 2020). The electrons for the reaction were provided by ferredoxin. In addition, it was found that the recombinant cyclase was only obtained in an active form when co-expressed with Ycf54 but Ycf54 itself did not seem to be required during catalysis (Stuart et al., 2020). Numerous in vivo studies have also demonstrated the importance of Ycf54 for the cyclase (Hollingshead et al., 2016; Chen et al., 2017; Chen et al., 2018; Chen and Hunter, 2020). In barley (Hordeum vulgare L.), the catalytic subunit of the cyclase is the XanL protein encoded by the Xantha-l gene. Three barley mutants, xan-l.35, xan-l.81, and xan-l.82 which are defective in the cyclase reaction due to mutations in the Xantha-l gene have been described previously (Rzeznicka et al., 2005). In addition, two mutants in the Viridis-k gene, vir-k.23 and vir-k.170 (Figure 2), have been isolated (Simpson and von Wettstein, 1980) and determined to be deficient in the cyclase reaction (Steccanella et al., 2015). Similar to the xantha-l mutations, the viridis-k mutations are lethal and have to be maintained in heterozygous stocks. Previous studies have established that the Viridis-k gene is clearly different from the Xantha-l gene and that it does not code for Ycf54 (Bollivar et al., 2014). We therefore set out to identify the Viridis-k gene to determine what role this additional component has in the cyclase reaction. As it turns out, the Viridis-k gene encodes the barley ortholog of Arabidopsis FdC2.
RESULTS

Viridis-k is located on chromosome 4H

In order to map the location of the Viridis-k gene we constructed an F2-mapping population by crossing the mutant vi√-k.23 to the barley cultivar Quench. Since nothing was known previously about the location of the Viridis-k gene in the barley genome, we used a genotyping-by-sequencing approach on the F2-mapping population to obtain single-nucleotide polymorphism (SNP) genotypes distributed throughout the whole genome. We sequenced 30 F2 individuals (Supplemental Table S1) and obtained approximately 700 million paired-end reads in total. Raw reads were demultiplexed to assign reads to the correct individuals and to remove reads with errors in the barcode or the restriction enzyme recognition sequence. Overall, roughly 90% of reads were retained after demultiplexing. The analysis of SNPs showed a single region associated with the viridis-k phenotype on the short arm of chromosome 4H (Figure 3) which is expected from a trait that is controlled by a single locus. This region was approximately 9.6 Mbp long and located between base pair positions 6.5-16.1 Mbp on chromosome 4H. The region showed complete linkage to the viridis-k phenotype (Figure 2) and thus represented the interval where the Viridis-k gene is located.

Identification of a Viridis-k candidate gene

In order to narrow down the interval where the Viridis-k gene is located a larger F2 population of roughly 300 individuals from the vi√-k.23 x Quench cross were genotyped with Cleaved Amplified Polymorphic Sequences (CAPS) markers (Supplemental Table S2) designed for SNPs discovered during genotyping-by-sequencing. The recombination frequency between each marker and the Viridis-k locus was used to calculate the genetic distance measured in centi-Morgans (cM) between the marker and the vi√-k.23 mutation. This mapping was able to narrow down the interval to between 8.7-16.1 Mbp on chromosome 4H representing a region of about 7.4 Mbp. The two flanking markers located at 8.7 Mbp and 16.1 Mbp were calculated to be located at a genetic distance of 6.7 cM and 2.1 cM away from the Viridis-k gene, respectively. Surprisingly, a dramatic drop in recombination rate was observed that resulted in markers at 9.7 Mbp, 10 Mbp, and 13.7 Mbp showing a genetic distance of 0 cM to the Viridis-k gene (Supplemental Table S3). This region contained 149 high confidence genes (Supplemental Data...
Set S1). The best candidate gene in the interval was the ferredoxin orthologous to Arabidopsis
FdC2. Since the cyclase is a ferredoxin dependent enzyme there is a direct functional link
between FdC2 and the biochemistry of the cyclase reaction (Stuart et al., 2020). We therefore
investigated if the barley gene orthologous to FdC2 contained any mutations in the two available
viridis-k mutants.

Identification of genetic changes in viridis-k mutants
Sanger sequencing identified a single base pair substitution, a G to A transition, in the vir-k.170
mutant. The transition changed a GCC codon to an ACC codon, which results in an alanine to
threonine substitution (Ala-118-Thr) (Figure 4). The G to A transition is consistent with the
types of mutations that have previously been reported in barley after sodium azide mutagenesis
(Olsen et al., 1993), which is the treatment used to generate vir-k.170. In the vir-k.23 mutant the
gene failed to be amplified by PCR using primers at the extreme ends of the gene but the 5’ and
3’ portions could be amplified separately (Figure 5). This suggested that the vir-k.23 mutation is
a larger chromosomal rearrangement which is consistent with the possible mutations induced by
X-rays (Randolph, 1950; Hagberg and Tjio, 1951).

To further characterize the mutants, we performed a reverse-transcription PCR (RT-PCR)
analysis of the FdC2 ortholog in vir-k.23 and vir-k.170 as well as the control Bonus, which is the
cultivar that the mutants were induced in. Since the vir-k.23 mutant appeared to have a
chromosomal break in the middle of the gene, we designed two primer pairs, one pair on either
side of the hypothesized break, to test for the presence of transcripts. This revealed that the 5’
portion of the gene was transcribed in both the mutants as well as the control. However, no
transcript was detected covering the 3’ portion in the vir-k.23 mutant even though this region
could be amplified from genomic DNA and must therefore be present somewhere in the genome.
The vir-k.170 mutant and Bonus accumulated RNA transcribed from the 3’ portion of the gene as
expected (Figure 5).

The viridis-k phenotype can be complemented in vivo by Viridis-k but not by Fd1
To further confirm that the viridis phenotype was indeed due to disruption of the identified gene
we set out to perform in vivo genetic complementation. Due to the lengthy process and genotype
specific requirements of performing stable transformations in barley (Harwood, 2014), we employed a transient expression approach based on agroinfiltration. This technique, has to the best of our knowledge, not previously been used to complement chlorophyll deficient mutants. To do this, we cloned full length Viridis-k from cDNA obtained from the barley cultivar Bonus and inserted in a plant overexpression vector for Agrobacterium tumefaciens mediated transformation. As a control, the canonical photosynthetic ferredoxin Fd1 was cloned into the same vector. The transient expression resulted in chlorophyll formation around the site of infiltration when Viridis-k was used but not Fd1. The successful complementation was best visualized with an imaging-PAM to reveal photosystem II chlorophyll fluorescence (Figure 6).

This clearly showed presence of photosystem II bound chlorophyll, thus confirming that the suggested candidate gene, orthologous to Arabidopsis FdC2, is deficient in the viridis-k mutants and that Viridis-k is required for proper accumulation of chlorophyll in vivo. A search through the barley genome revealed eight plastid localized ferredoxins that are transcribed in the nucleus (Figure 7) (Mayer et al., 2012; Colmsee et al., 2015; Mascher et al., 2017; Monat et al., 2019). The chlorophyll deficient phenotype of viridis-k mutants and the lack of complementation when leaf-type Fd1 was used for agroinfiltration suggest that Viridis-k has a specific role that cannot be adequately performed by other ferredoxins in vivo.

**Presence of cyclase components in known mutants**

Immunoblot analysis was performed on total protein extracts from barley cyclase mutants, xan-l.35, xan-l.81, and xan-l.82 as well as vir-k.23 and vir-k.170. The xan-l.35 mutant has a point mutation and a leaky phenotype producing small amounts of chlorophyll. The xan-l.81 and xan-l.82 mutants are both completely blocked at the cyclase step and have a single amino acid substitution and introduction of an early stop codon, respectively (Rzeznicka et al., 2005). Blots with antibodies against XanL showed a dramatic decrease of XanL in xan-l.35, while no XanL accumulated in xan-l.82. The vir-k.23 and vir-k.170 mutants accumulated XanL to similar levels as the control cultivar Bonus (Figure 8A). The same pattern was observed for immunoblots probed with antibodies raised against the cyclase associated protein Ycf54, which shows that Ycf54 does not accumulate in the absence of XanL and that the levels of Ycf54 increase with increasing levels of XanL (Figure 8A). Immunoblots using an antibody that recognizes canonical ferredoxin shows presence in all lines. However, immunoblots using antibodies raised against
VirK showed that no VirK protein accumulates in the \textit{vir-k.23} mutant, while trace amounts were seen in the \textit{vir-k.170} mutant (Figure 8A). Interestingly, VirK accumulation also increases with increasing XanL accumulation in the \textit{xantha-l} mutants. That is, VirK decreased to hardly detectable levels in \textit{xan-l.35} and failed to accumulate in the \textit{xan-l.82} mutant which lacks XanL. The effect is apparently posttranslational since quantitative reverse-transcription PCR (RT-qPCR) analysis showed similar levels of \textit{Viridis-k} mRNA in all mutants except \textit{vir-k.23}, which has a large chromosomal rearrangement (Figure 8B). That VirK accumulation is apparently dependent on cellular accumulation of XanL indicates an important \textit{in vivo} interaction.

\textbf{VirK can function as an electron donor to the cyclase reaction}

Since the XanL is a ferredoxin dependent enzyme and the VirK isoform is clearly associated with the cyclase reaction \textit{in vivo}, it seems likely that VirK is the main electron donor to the enzyme. To test if VirK could provide electrons to drive the cyclase reaction we produced recombinant VirK and used it as the electron donor for cyclase enzyme activity assays using recombinant XanL. The results clearly showed that VirK can drive the cyclase reaction with electrons provided by NADPH via FNR (Figure 9). Formation of the product, protochlorophyllide, increased with increasing VirK concentrations until saturation was reached, clearly showing that VirK can donate electrons to the cyclase enzyme. No product was formed if VirK was omitted from the reaction.
DISCUSSION

Plants and other photosynthetic organisms have a rich variety of ferredoxins. A search of the barley nuclear genome revealed eight ferredoxins with a chloroplast transit peptide. A comparison of these to ferredoxins of maize (*Zea mays* L.) and Arabidopsis as well as the green alga *Chlamydomonas reinhardtii* and the cyanobacterium *Synechocystis* PCC6803 shows three classes of ferredoxins (Figure 7). Both of the plant ferredoxin isoforms with C-terminal extensions, FdC1 and FdC2, form well supported sub-trees. The FdC2 sub-tree contains VirK as well as orthologs in all species including *Synechocystis*. This is consistent with a highly conserved and ancient function which is supported by the essential nature of the gene in plants as well as cyanobacteria (Cassier-Chauvat and Chauvat, 2014; Schorsch et al., 2018). The FdC1 subtree does not contain a representative from *Synechocystis* but the Chlamydomonas FDX4 ortholog clusters into this group.

The canonical ferredoxin isoforms from plants were first characterized as “photosynthetic” and “non-photosynthetic” but now are generally referred to as “leaf-type” and “root-type”, respectively. The separation into different categories was based on early studies which found that maize contains isoforms located primarily in green tissue (FdI and FdII) which are regulated by light and an isoform found primarily in roots (FdIII) where photosynthesis does not occur (Hase et al., 1991). Another apparent difference between these two categories is that the leaf-type ferredoxins generally have a more negative redox potential than the root-type. Thus, the midpoint potential for NADPH is in-between that of leaf- and root-type ferredoxins. The midpoint potential of ferredoxin isoforms is likely an important biochemical property for predicting *in vivo* function since leaf-type ferredoxins are reduced by photosystem I in order to reduce NADP* to NADPH via FNR while root-type ferredoxins are reduced by NADPH via FNR to support ferredoxin dependent metabolism in the absence of photosynthesis (Shinohara et al., 2017). The leaf-type ferredoxins form a clear subtree and both Arabidopsis and barley have two apparent leaf-type ferredoxins. Maize has four leaf-type ferredoxins, which is likely due to a need for additional specialization since maize has a C4 metabolism. Consistent with this, FdI and FdII have been previously characterized as being specific for bundle sheath cells and mesophyll cells, respectively (Kimata and Hase, 1989; Matsumura et al., 1999). Arabidopsis contains, in
addition to the two leaf-type ferredoxins, two more canonical ferredoxins which were classified as a root-type and an “other” type based on divergent sequence and a much higher redox potential than the root-type (Hanke et al., 2004). Both of these fall into the same subtree which also contains three maize ferredoxins including the root-type. Barley also has three ferredoxins in this subtree. A third subtree separate from the leaf- and root-type ferredoxins contains only representatives from barley and maize, both of which are monocots. One of these, maize FdVI, has been shown to be induced in roots by nitrate (Matsumura et al., 1997). Ferredoxins in this subtree may thus have a monocot specific role in nitrogen assimilation.

The Chlamydomonas and Synechocystis ferredoxins, except for SynFed3, are clearly most closely related to canonical plant ferredoxins although they are divergent enough to not cluster within the leaf- and root-types. In Chlamydomonas the main photosynthetic ferredoxin is PETF/FDX1 that has a midpoint potential of -398 mV (Terauchi et al., 2009) which is similar to that of Arabidopsis and maize leaf-type ferredoxins which range from -433 mV to -406 mV (Hase et al., 1991; Hanke et al., 2004). The Chlamydomonas FDX2 is most closely related to PETF/FDX1 but has a midpoint potential of -321 mV (Terauchi et al., 2009) which is similar to that of Arabidopsis and maize root-type ferredoxins which have midpoint potentials of -337 mV and -321 mV respectively (Hase et al., 1991; Hanke et al., 2004). Based on the midpoint potentials, FDX2 may have similar functions to plant root-type ferredoxins in supporting ferredoxin dependent metabolism in the absence of photosynthesis.

Mutants are invaluable tools for revealing molecular processes in vivo. The yellow mutants of vascular plants are chlorophyll deficient and have been explored to learn about genes and enzymes involved in chlorophyll biosynthesis. The biosynthetic step inhibited by a mutation is generally identified by accumulation of the substrate of the affected enzyme. However, a feedback mechanism prevents accumulation of chlorophyll biosynthetic intermediates unless the plants are fed with 5-aminolevulinic acid, which is the common precursor after the main regulatory step of the pathway. In this way, barley chlorophyll mutants were assigned to specific steps in the chlorophyll biosynthetic pathway (Gough, 1972). The vir-k.23 and vir-k.170 mutants were shown to be deficient in the cyclase reaction (Steccanella et al., 2015). In the present study, we identified the Viridis-k locus as the gene orthologous to Arabidopsis FdC2, Chlamydomonas
FDX6 and *Synechocystis SynFed2* encoding one of the two ferredoxin isoforms with C-terminal extension. Genetic mapping data identified a region containing 149 high-confidence genes including an *FdC2* ortholog, which appeared as the top candidate for several reasons. First, the known cyclase components are located in the chloroplast. Therefore, the gene product of the candidate gene should be localized to the chloroplast. The FdC2 ortholog was one of 32 gene products in the mapping interval with a predicted chloroplast transit peptide. Second, the cyclase reaction requires reducing power and was recently demonstrated to be a ferredoxin dependent enzyme (Stuart et al., 2020) making the FdC2 ortholog the only chloroplast targeted candidate with an obvious functional association to the biochemistry of the cyclase enzyme. Previously, two mutants in the orthologous *FdC2* gene in rice had been isolated and were pale green due to decreased chlorophyll accumulation (Li et al., 2015; Zhao et al., 2015) but no further characterization or suggestions to their involvement in the cyclase reaction was reported. In addition, one would expect a gene required for chlorophyll biosynthesis to be highly conserved among oxygenic photosynthetic organisms. Recent studies on the evolution of photosystem I electron acceptors show that FdC2 orthologues are conserved and found in all green plastids (green algae and land plants) as well as cyanobacteria from which chloroplasts derive (Karpowicz et al., 2011; Pierella Karlusich et al., 2015; Pierella Karlusich and Carrillo, 2017).

Sanger sequencing identified a single base pair mutation resulting in an Ala-118-Thr mutation in *vir-k.170*. The change from alanine to threonine is a drastic change since alanine is a small non-polar amino acid while threonine is bulkier and polar. In addition, Ala-118 is highly conserved across C-type ferredoxins from a range of species including land plants, green algae, and cyanobacteria (Figure 4) which indicates that substitution of this residue is not well tolerated.

The *vir-k.23* mutant has a lesion in the *Viridis-k* gene. The mutation is consistent with a large-scale genomic rearrangement such as an inversion, a large insertion or a translocation spanning several million base pairs and that one of the break points is within the *Viridis-k* gene. The 5’ and 3’ regions of the gene could be amplified by PCR in the *vir-k.23* mutant from genomic DNA but amplification across the central portion was not possible in the mutant while it was possible in the control cultivar Bonus, as well as in *vir-k.170*. This indicates that both ends of the gene are present in the genome but that they are no longer positioned such that they are in the proper
orientation or appropriate distance from each other to allow amplification by PCR or production of a full-length transcript. Combined with the CAPS mapping data of viridis-k.23 which showed a drop in the recombination frequency to zero over about 4 Mbp, the most likely scenario is a large-scale genomic rearrangement which would be consistent with the mutagen used since ionizing radiation such as X-rays are known to be able to induce double stranded DNA breaks (Randolph, 1950; Hagberg and Tjio, 1951). The presence of a lesion in the middle of the gene was also supported by RT-PCR which showed that the vir-k.23 mutant accumulates transcripts from the 5’ portion of the gene but not from the 3’ portion. Immunoblots against VirK show that the protein does not accumulate in the vir-k.23 mutant which is expected considering that the gene is disrupted by a large structural mutation. While not completely absent, VirK accumulation was reduced in the vir-k.170 mutant, which suggests that the Ala-118-Thr substitution affects the in vivo stability of the protein. The mutation could affect either the physical stability of the protein and/or make it more prone to degradation by proteases. The level of Viridis-k mRNA in vir-k.170 was not different from the mother cultivar Bonus (Figure 8).

Identification of multiple independent alleles is an efficient approach to validate a candidate gene. Over the years we have successfully used this approach where up to 215 alleles have been available; the Eceriferum-cqu locus involved in synthesis of epicuticular waxes as an example (Schneider et al., 2016). Unfortunately, only two viridis-k mutant alleles are available. To further support that we had identified the mutated gene in viridis-k mutants, we performed a transient expression of Viridis-k in seedling leaves of vir-k.23 mutants. A. tumefaciens containing barley Viridis-k was infiltrated into the leaves. Typically, Agrobacterium-mediated infiltration is performed in dicot plants (Kapila et al., 1997; Wroblewski et al., 2005). After 5-7 days post infiltration, chlorophyll formation could be seen around the site of infiltration conclusively demonstrating that the correct gene had been identified.

The viridis-k mutants are yellow, but trace amounts of chlorophyll can be detected (Steccanella et al., 2015). It is likely that this small amount of chlorophyll can be synthesized due to nonspecific action of other ferredoxins in the vir-k.23 and vir-k.170 mutants where VirK is absent or inactivated. Redundant cyclase activity supported by other ferredoxins might be expected since we recently showed that even leaf-type ferredoxin from spinach can drive the
aerobic cyclase reaction *in vitro* using barley XanL (Stuart et al., 2020). Further, the aerobic cyclase, AcsF, of the purple bacterium *Rubrivivax gelatinosus* can be driven by ferredoxin from spinach and *Anabaena* (Chen et al., 2021). However, the *viridis*-k mutations are lethal and homozygous mutant seedlings die after approximately two weeks when the starch in the kernel is depleted. Thus, VirK plays a vital role in chlorophyll biosynthesis and its function cannot be meaningfully replaced by other ferredoxins *in vivo*. Additionally, *Viridis*-k is tightly connected to the cyclase enzyme system since we have shown that the steady state level of VirK is dependent on the level of XanL. This suggests an intimate connection between XanL and VirK *in vivo*. The same phenomenon has been observed previously with other enzymes involved in chlorophyll biosynthesis. For example, the magnesium chelatase, which inserts Mg$^{2+}$ into protoporphyrin IX consists of three subunits encoded in barley by *Xantha*-h, *Xantha*-g, and *Xantha*-f (von Wettstein et al., 1974; Jensen et al., 1996). The *xantha*-h mutants that do not produce a XanH protein also fail to accumulate the XanG protein which is known to interact directly with XanH (Hansson et al., 1999; Lake et al., 2004; Lundqvist et al., 2010). The decreased accumulation of VirK is unlikely to be an indirect effect due to the lack of chlorophyll biosynthesis since VirK accumulates in the *xan-l.81* mutant which is also completely blocked at the cyclase step but does accumulate XanL. In contrast, the *viridis*-k mutants accumulate canonical ferredoxins but still have a chlorophyll deficient phenotype, and the *vir-k.23* mutant could be complemented by introducing *Viridis*-k but not leaf-type *Fd1*. Taken together with the fact that XanL is a ferredoxin dependent enzyme and that VirK can donate electrons to XanL *in vitro*, it is likely that VirK is the electron donor to the cyclase reaction *in vivo* and that the other ferredoxin isoforms offer little redundancy when it comes to supplying electrons to the cyclase enzyme.

An alternative function of *Synechocystis* Fed2 has recently been suggested (Schorsch et al., 2018). In their study, SynFed2 was suggested to be involved in iron homeostasis. An explanation consistent with their data and the results presented in this paper is that the observed phenotypes are due to a deficiency in chlorophyll biosynthesis. The cyanobacterial study made use of C-terminal truncations since complete deletion of the gene was lethal. In the absence of a complete loss of function mutant in cyanobacteria it is difficult to directly compare results, but the truncation mutants showed decreased growth and lower levels of chlorophyll accumulation.
Since XanL is an iron containing enzyme, one might speculate that the cyclase deficient phenotype in the *viridis*-k mutants is due to improper assembly of the diiron cluster of XanL. Improperly matured proteins tend to be degraded by the cell and this seems to be the case for improperly matured cyclase (Albus et al., 2012; Hollingshead et al., 2016; Chen and Hunter, 2020; Stuart et al., 2020). If the *viridis*-k mutations resulted in improper maturation of XanL we would expect to see decreased XanL levels in the *viridis*-k mutants which was not observed. In fact, the opposite was found – XanL is required for proper accumulation of VirK. Additionally, there was no apparent deficiency in the accumulation of canonical ferredoxin in the mutants which could also be expected if iron homeostasis was perturbed. The data is thus inconsistent with improperly matured XanL in the *viridis*-k mutants but is consistent with a function for VirK as the specific *in vivo* electron donor to the cyclase reaction.
METHODS

Plant Material
The barley (*Hordeum vulgare* L.) vir-k.23 mutant was isolated in 1954 by x-ray mutagenesis of the cultivar Svalöv Weibull’s Bonus (Simpson and von Wettstein, 1980). The xan-l.35 mutant was isolated in 1957 by ethyleneimine mutagenesis of Bonus while xan-l.81 and -l.82 mutants were isolated in 1975 by sodium azide mutagenesis of the *tigrina-d.12* mutant (Bonus genetic background) and screening for mutants that accumulated protochlorophyllide. The mutants were then backcrossed to Bonus in order to remove the *tigrina-d.12* mutation (Henningsen et al., 1993). Also in 1975, another mutant accumulating protochlorophyllide, originally named *xantha-83* was isolated which later turned out to be allelic to *vir-k.23* and thus renamed to *vir-k.170* (personal communications, Diter von Wettstein).

Mapping Population
An F₂ mapping population was constructed by crossing the *vir-k.23* mutant to the cultivar Quench as the male parent. Since the *vir-k.23* mutation is a recessive lethal mutation the female parent plants used were green and could be either homozygous wild type or heterozygous for the *vir-k.23* mutation. Due to this, some F₁ plants were homozygous wild type and their offspring could not be used for mapping. To eliminate these individuals, the seeds of F₁ plants were harvested on a per plant basis and one spike from each plant was grown in moist vermiculite for 9 days under a lab bench away from direct sunlight to check for segregation of the *vir-k.23* phenotype. Only seeds from F₁ plants whose offspring segregated were kept for the mapping population. The F₂-mapping population was grown under a lab bench away from direct sunlight. F₂-plant material was collected after 9 days.

Genomic DNA Isolation
Genomic DNA for genotyping-by-sequencing library preparation or to be used as template for PCR was isolated by a modified CTAB protocol (Doyle, 1991). Between 100-200 mg leaf material was frozen under liquid nitrogen in screw cap tubes with two 4 mm glass beads and subsequently homogenized on a FastPrep 24 (MP Biomedicals) for 4x 15 s at 4 m/s. Homogenized plant material was mixed with 1 mL 2x CTAB buffer (2% [w/v]
cetyltrimethylammonium bromide, 200 mM Tris-HCl pH 8, 20 mM EDTA, 1.4 M NaCl, 1%
[w/v] polyvinylpyrrolidone (40 g/mol), 0.28 M β-mercaptoethanol) and incubated at 65°C for 30
min after which 800 µl chloroform:isoamylalcohol (24:1 [v/v]) was added and the samples were
mixed on a rotary shaker for 15 min at room temperature. Samples were then centrifuged at
6,200 x g for 30 min at 4°C in order to separate the phases and 800 µL of the aqueous phase was
transferred to a new tube and incubated at 37°C for 15 min with 50 µg RNase A. DNA was
precipitated by addition of 560 µL isopropanol followed by centrifugation for 15 min at 15,700 x
g and 4°C. The resulting pellet was then washed for 15 min in 76% (v/v) ethanol with 200 mM
sodium acetate followed by a 5 min wash in 76% (v/v) ethanol with 10 mM ammonium acetate.
After washing, the DNA pellet was allowed to dry at room temperature for 10 min prior to

suspension in Low TE (10 mM Tris-HCl pH 8, 0.1 mM EDTA).

**Genotyping-by-Sequencing Library Preparation**

Genotyping-by-sequencing library preparation for 30 F₂ seedlings was performed essentially as
previously described (Poland et al., 2012) with minor modifications and using adaptor sequences
as described in (Baird et al., 2008) with the modification that the P2 adaptors were modified to
be compatible with MspI digested DNA ends. Briefly, 200 ng gDNA per individual was digested
with 8 units PstI-HF (New England Biolabs) and 8 units MspI (New England Biolabs) in 1x
NEB buffer 4 (New England Biolabs) by incubation for 2 h at 37°C. Sample specific inline
barcoded P1 adaptors as well as a common P2 Y-adaptor that have overhangs complementary to
PstI and MspI, respectively, were then ligated to the digested gDNA using 200 units T4 DNA
ligase (New England Biolabs) in a reaction mixture containing all 200 ng restriction digested
gDNA, 1 mM ATP, 2.5 nM P1 adaptor, and 375 nM P2 adaptor in 1x NEB buffer 4. The ligation
reaction was incubated for 2 h at 22°C. At this point samples were pooled and concentrated to 60
µL using a Qiagen Quiaquick PCR purification spin column (Qiagen) following manufacturer
instructions. Next, 20 µL was used as template for a 200 µL PCR, split into 8x 25 µL reactions
using NEB NEXT Q5 Ultra II master mix (New England Biolabs). PCR enriches for DNA
fragments containing both P1 and P2 adapters. Splitting of the PCR reaction mixture into
multiple smaller reactions is done to minimize bias introduced by stochastic effects of PCR.
After PCR the reactions were pooled and concentrated to 30 µL using a QiaQuick spin column
(Qiagen) and run on a 1.25% (w/v) agarose 1x TAE gel at 100 volts for one h. The region
containing molecules in the size range of 450-650 bp was cut out in order to size select the DNA fragments. DNA was purified from the excised gel using a JetSorb gel extraction kit (Genomed). The JetSorb gel extraction was performed according to the manufacturer’s instructions with the modification that two extra washes with 80% (v/v) ethanol and 10 mM Tris-HCl pH 7.5 were added and the DNA was eluted in 60 μL Qiagen buffer EB. Sequencing was performed on an Illumina HiSeq 2500 with 125 cycles of paired end sequencing with Illumina v4 chemistry.

Genotyping-by-Sequencing Data Analysis
Raw reads were demultiplexed using the process_radtags component of the STACKS (v.1.40) pipeline (Catchen et al., 2011). Phenotypes and index sequences for the F2 individuals in the sequencing libraries can be found in Supplemental Table S1. Process_radtags produced 4 files for each individual, two files with paired reads, and two files with unpaired read. The 4 files for each individual were combined into a single file for each individual. Reads were then aligned to the barley reference genome (Mascher et al., 2017) using BWA mem (v.0.7.13) to produce sam files (Li and Durbin, 2010). Reads aligned to the reference genome were then further processed through the stacks pipeline to output a list of SNPs in variant call format (.vcf).

The vcf output file was further analyzed using TASSEL (v.5.2.31) in order to locate SNPs that were associated with the viridis phenotype in our mapping population (Bradbury et al., 2007). The vcf file was loaded in TASSEL and sorted followed by filtering SNPs such that they needed to be present in 20 individuals and have a minimum allele frequency of 0.4 and a maximum allele frequency of 0.6 (an allele has an expected frequency of 0.5 in an F2 population). Next, numerical values for phenotypes were assigned to the plants by setting wild type to “0” and mutants to “100”. The General Linear Model (GLM) analysis was run with default settings except that “run permutations” was set to 10 and “Bi-allelic sites only” was selected since a SNP should have only two alleles in the mapping population. The output from the GLM analysis was then visualized to identify the genomic region linked to the mutant phenotype.

CAPS Genotyping and Genetic Mapping
Additional F2 individuals were genotyped using Cleaved Amplified Polymorphic Sequence (CAPS) markers designed for SNPs discovered during genotyping-by-sequencing. For all markers PCR was performed in 20 μL reactions using RedExtract-N-Amp (Sigma) following...
manufacturer’s instructions. PCR product was then digested with restriction enzymes (New England Biolabs) specific for one allele of the SNP to be analyzed followed by separation on a 2% (w/v) agarose 1x TAE gel run for 22 min at 100 volts. PCR primers used can be found in Supplemental Table S2. Five CAPS markers were tested located on chromosome 4H at 8.7 Mbp (4 units XbaI, Quench allele uncut), 9.7 Mbp (1 unit BtsCI, Quench allele cut), 10 Mbp (1 unit NcoI-HF, Quench allele cut), 13.7 Mbp (1 unit DdeI, Quench allele uncut), and 16.1 Mbp (1 unit NheI, Quench allele uncut). The recombination frequency between the SNPs and the viridis-k phenotype was calculated using a maximum likelihood approach (Fisher and Balmukand, 1928) and converted to genetic map units (cM) using the Kosambi mapping function (Kosambi, 1943).

**PCR and Sequencing of the Viridis-k Gene**

Sequencing of the Viridis-k gene was performed on overlapping PCR amplicons by Eurofins Genomics on an ABI 3730XL DNA Analyzer. PCR was run with either Phusion high fidelity DNA polymerase (New England Biolabs) or RedExtract-N-Amp Plant Tissue PCR Kit (Sigma) following manufacturers guidelines. The PCR products were cleaned up using Illustra ExoProStar 1-Step (Cytiva) following manufacturer’s instructions except if unspecific bands were produced in which case the PCR products were run on a 2% (w/v) agarose 1x TAE gel for 30 min at 100 volts. The correct bands were then cut out of the gel and cleaned up with NucleoSpin Gel and PCR clean-up columns (Macherey-Nagel) before sequencing.

**RNA Isolation, RT-PCR and RT-qPCR**

Plants were grown in vermiculite for 9 days under a lab bench away from direct sunlight and 300 mg material from a single leaf was harvested and immediately frozen in liquid nitrogen. Leaf material was homogenized under liquid nitrogen in a porcelain mortar. After homogenization total RNA was isolated using TRIzol reagent (Invitrogen). Residual DNA was removed by treating total RNA preparations with DNase I (Thermo Scientific) after which cDNA was synthesized using 0.5 µg RNA per 20 µL reaction using RevertAid Reverse transcriptase (Thermo Scientific). The cDNA from vir-k.23, vir-k.170 and Bonus were used for RT-PCR to detect HvFdC2 transcripts and cDNA from Bonus was used for cloning of wild type genes used in other experiments using Phusion high fidelity DNA polymerase (New England Biolabs).
For gene expression analysis using RT-qPCR, one leaf of three different plants was collected for RNA extraction per each biological replicate. Three technical replicates were performed on each biological sample. Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. RNA samples were treated by DNase on the columns using RNase-Free DNase Set (Qiagen). RNA integrity was checked on a 2% (w/v) agarose gel. The concentration and purity of the extracted RNA was measured spectrophotometrically. cDNA was synthesized from 1 µg of total RNA using the RevertAid First Strand cDNA synthesis kit (Thermo Scientific) following the manufacturer's instructions. Transcript levels were measured by using the SsoAdvanced™ Universal SYBR Green Supermix (Bio-Rad). Each reaction contained 20× diluted cDNA and primers at a final concentration of 0.25 µM. Samples were run on a CFX384TM Real-Time System (Bio-Rad) using the following thermal cycling conditions: 95°C for 2 min, followed by 40 cycles of 95°C for 10 s and 60°C for 10 s. The used pair of primers amplified the 5' region of the Viridis-k transcript (Supplemental Table S2). For normalization, the housekeeping gene E3 ubiquitin-protein ligase UPL6 gene (HORVU1Hr1G023480) was used. Results were expressed using the ΔCt calculation method (Schmittgen and Livak, 2008).

In Vivo Genetic Complementation by Agroinfiltration

Full length coding sequences of Viridis-k (MLOC_37911) and Fd1 (MLOC_6135) were amplified from cDNA and cloned into the overexpression vector pK7WG2 (Karimi et al., 2002) by Gateway cloning (Invitrogen) to generate pK7WG2HvFdC2 and pK7WG2HvFd1. The pK7WG2 constructs were transformed into Agrobacterium tumefaciens strain AGL0. Seeds of vir-k.23 were planted in vermiculite, watered and placed in a cold room (4°C), giving the seeds time to imbibe water and thereby synchronize germination. After 48 h the seeds were moved to a climate chamber (16/8 h of light/dark, 26.5°C, 35% relative humidity and 8,900 Lux). Bacteria were inoculated into LB media (10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl, pH 7) containing streptomycin (2 mg/mL). Cultures were incubated at 25°C with shaking for 48 h. New cultures were prepared by transferring bacteria from the two start cultures to two new cultures to OD_{600} of 0.1 in LB media containing streptomycin (2 mg/mL) and acetosyringone (200 µM). Cultures were incubated overnight at 25°C with shaking. Bacteria were pelleted by centrifugation (7,400 × g for 40 min). Supernatants were discarded and pellets were resuspended.
in MM buffer (10 mM 2-(N-morpholino) ethanesulfonic acid (MES) pH 5.7 and 10 mM MgCl₂) containing 200 μM acetosyringone. The bacteria were washed by a second centrifugation (7,400 × g for 40 min). Supernatant was discarded and pellet resuspended to an OD₆₀₀ of 1.1 in MM buffer containing 300 μM acetosyringone. Cultures were then incubated for 1-2 h at room temperature. Ascorbic acid to 20 mM was added to cultures immediately prior to infiltration. Bacteria were delivered into the abaxial side of the leaf by first creating a small scratch with a needle and then pressure infiltrating bacteria with a needleless syringe. Infiltrated seedlings were placed in dark overnight, under a plastic hood to maintain humidity. The next day the infiltrated seedlings were moved to a climate chamber (same settings as before) for visual observations.

**Chlorophyll Fluorescence detection**

Chlorophyll fluorescence was detected using an Imaging PAM M-series Maxi Version Chlorophyll Fluorometer (Heinz Walz). The leaves were dark-adapted for a minimum of 10 min before analysis. The leaves were cut off directly prior to measurements, placed with adaxial side up under microscope slides to be held in position. Data was analyzed using ImagingWin (v.2.47) software (Heinz Walz).

**Immunoblots**

Polyclonal rabbit antibodies against barley VirK were generated by Agrisera using recombinant VirK as the antigen. Other antibodies used were raised against barley proteins XanL (Bollivar et al., 2014), Ycf54 (Stuart et al., 2020) and purified ferredoxin (Andersen et al., 1992). Plants for total protein extraction were grown in vermiculite for 9 days under a lab bench away from direct sunlight and 200 mg tissue was snap frozen in liquid nitrogen in 2 mL screw cap vials with two 4 mm glass beads and 10 μL 2.5% (w/v) PMSF in isopropanol. Plant material was homogenized by grinding in a FastPrep 24 at 4 m/s for 10 s repeated 10 times with 4 min under liquid nitrogen between cycles to prevent material from thawing. Next, 500 μL protein extraction buffer (12 M urea, 2% [w/v] SDS, 100 mM DTT, 100 mM Tris-HCl pH 8, and 10 mM EDTA pH 8) was added, briefly heated to 60°C and mixed by running four more cycles in the FastPrep 24. Samples were then incubated at 99°C and 14,000 rpm for 30 min in a Thermomixer comfort (Eppendorf) followed by centrifugation for 10 min at 16,000 × g. The supernatant was transferred to fresh tubes and centrifuged for another 30 min. The final supernatant was saved as
the total protein extract. SDS-PAGE and immunoblots were performed as described previously (Stuart et al., 2020).

**Recombinant Protein Production and Cyclase Activity Assay**

Recombinant XanL was prepared as described previously (Stuart et al., 2020). The coding sequence of *Viridis-k* without the transit peptide as predicted by ChloroP (Emanuelsson et al., 1999) was codon optimized and cloned into pET15b by GenScript Biotech Corporation to generate pET15bHvFdC2. *E. coli* BL21(DE3) transformed with pET15bHvFdC2 was inoculated to an OD<sub>600</sub> of 0.1 in 250 mL LB containing 100 µg/mL ampicillin and placed in an incubator at 20°C and 200 rpm. After one h the temperature was set down to 15°C. Once the culture reached an OD<sub>600</sub> of 0.3, IPTG was added to a final concentration of 1 mM and solid FeSO<sub>4</sub> was added to a final concentration of 2 mM. After 24 h another 2 mM equivalent of solid FeSO<sub>4</sub> was added and the culture grown for an additional 36 h before the cells were harvested by centrifugation and stored at -80°C until use. For protein purification a cell pellet was resuspended to 50 mL in binding buffer (20 mM Tris-HCl pH 8, 500 mM NaCl, and 20 mM imidazole, 5 mM DTT) supplemented with 3 M urea as well as a few grains of DNase I and lysozyme. The cell suspension was passed through a french press three times at 12.4 MPa after which the lysate was centrifuged 10 min at 48,384 × g. The supernatant was loaded on two 1 mL HisTrap FF crude (Cytiva) columns connected in series and washed 4 times with 15 mL binding buffer supplemented with decreasing concentrations of urea. The urea concentration was decreased from 3 M to no urea in 1 M increments. The column was then washed with 15 mL wash buffer (20 mM Tris-HCl pH 8, 500 mM NaCl, 40 mM imidazole, and 5 mM DTT) followed by elution with 20 mM Tris-HCl pH 8, 500 mM NaCl, 750 mM imidazole, and 5 mM DTT. The VirK protein was desalted over a NAP-10 (Cytiva) column into 50 mM Tris-HCl pH 8 with 1 mM DTT, after which 87% (v/v) glycerol was added to a final concentration of 15% (v/v). The VirK protein was aliquoted and stored at -80°C until use.

The coding sequence of barley root isoform of FNR (HvRFNR, MLOC_6838) was cloned from cDNA without the chloroplast transit peptide as predicted by ChloroP into the vector pDEST17 by Gateway cloning to produce pDEST17HvRFNR. *E. coli* Rosetta (DE3)pLysS transformed with pDEST17HvRFNR was inoculated to an OD<sub>600</sub> of 0.1 into 250 mL LB supplemented with 100 µg/mL ampicillin and 25 µg/mL chloramphenicol and grown at 25°C and 200 rpm until the
culture reached an OD$_{600}$ of 0.4, after which protein expression was induced by adding IPTG to a final concentration of 1 mM. Cells were harvested by centrifugation the next morning after 12-16 h of growth post induction and cell pellets were frozen at -80°C until use. A cell pellet corresponding to 250 mL culture was resuspended to 20 mL in binding buffer supplemented with 100 µg/mL lysozyme after which cells were disrupted by sonication on ice for a total of 4 min at 50% output. The lysate was centrifuged 8 min at 48,384 × g, after which the supernatant was loaded onto a 1 mL HisTrap FF crude column. The column was washed with 5 mL binding buffer followed by 10 mL wash buffer. The recombinant HvRNFR was eluted with 20 mM Tris-HCl pH 8, 500 mM NaCl, 250 mM imidazole, and 5 mM DTT and desalted over a NAP-10 column into buffer consisting of 1 mM DTT, 25 mM MgCl$_2$, 1 mM EDTA, 20 mM Tricine, 10 mM HEPES, and adjusted to pH 8.1 with NaOH. After desalting, 87% (v/v) glycerol was added to a final concentration of 15% (v/v) and the protein was aliquoted and frozen at -80°C until use.

Cyclase assays were run as described previously (Stuart et al., 2020) except that the above recombinant HvRFN and VirK were used instead of spinach FNR and ferredoxin from Sigma. MPE was extracted from a bchE mutant of Rhodobacter capsulatus as previously described (Gough et al., 2007). A measurement series consisted of six enzymatic reactions with different amounts (0-70 µg) of added VirK but constant amounts of XanL (40 µg) and HvRNFR (13 µg) in total volumes of 30 µL containing 20 mM Tricine and 10 mM HEPES pH 8.1, 1 mM EDTA, 25 mM MgCl$_2$, 1 mM DTT, 10 mM glucose-6-phosphate, 0.03 units/µL glucose-6-phosphate dehydrogenase, 0.5 mM NADPH, 90 µg/µL catalase, 0.026% (v/v) Triton X-100, and 10 µM MPE. Assays were incubated in the dark at 30 °C and 750 rpm for one hour in a Thermomixer comfort (Eppendorf). Assays were stopped by addition of 80% (v/v) acetone with 0.32% (v/v) NH$_3$ and centrifuged for 5 min at 29,000×g to pellet precipitated proteins. Formation of protochlorophyllide was measured using an RF-5301 PC spectrofluorometer (Shimadzu) with an excitation wavelength of 440 nm and an emission spectrum between 570 and 700 nm with slit widths of 10 nm for both excitation and emission. Product formation was estimated as the relative fluorescence emission at 634 nm for samples minus the emission for the negative control sample. Linear regression was performed with the lm() function in R (v.3.6.1).

Phylogenetic Analysis
Ferredoxin sequences for Arabidopsis, maize, Chlamydomonas and *Synechocystis* were retrieved by BLAST against the NCBI Protein reference sequences database with an E value cutoff of $10^{-20}$ for a match using each of the barley ferredoxins as queries. In order to avoid bias from erroneous prediction of chloroplast transit peptides all sequences were N-terminally trimmed to the shortest *Synechocystis* ferredoxin after removal of the initiator methionine as these do not provide a phylogenetic signal. Sequences were aligned using ClustalW as implemented in MEGA-X (v. 10.2.5). The alignment in FASTA format can be found in Supplemental File S1. MEGA-X was used to determine the best fitting maximum likelihood model after which a maximum likelihood tree was inferred using the LG +G model with 16 rate categories and gamma parameter equal to 1.6872. Node support was determined using 1000 bootstrap replicates. Nodes with less than 50% support were condensed in the bootstrap consensus tree. The tree was visualized as an unrooted tree using iTOL (Letunic and Bork, 2021). The tree in Newick format can be found in Supplemental File S2.

**Accession numbers**
Sequence data from this article can be found in the NCBI Sequence Read Archive ([https://www.ncbi.nlm.nih.gov/sra](https://www.ncbi.nlm.nih.gov/sra)) under accession no. PRJNA686392.

**Supplemental data**

**Supplemental Table S1.** Phenotype and index sequence for F$_2$ individuals included in the genotyping-by-sequencing libraries for mapping of the *Viridis*-k gene.

**Supplemental Table S2.** List of used DNA oligonucleotides.

**Supplemental Table S3.** Positions and genetic distances of markers to the *Viridis*-k gene.

**Supplemental Table S4.** Ct values from RT-qPCR of the 5’ region of the *Viridis*-k gene.

**Supplemental Table S5.** Ct values from RT-qPCR of the housekeeping gene E3 ubiquitin-protein ligase *UPL6* (HORVU1Hr1G023480).

**Supplemental Table S6.** Calculations of relative expression of *Viridis*-k in the five mutants and their mother cultivar Bonus.

**Supplemental Data Set S1.** List of genes in the mapped interval for *Viridis*-k.

**Supplemental File S1.** Multiple sequence alignment of ferredoxin peptide sequences used for constructing phylogenetic relations in Figure 7.
Supplemental File S2. Phylogenetic tree (Figure 7) in Newick format.

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Author contributions
D.S., D.B., M.H. and P.E.J. designed the experiments; D.S., H.M.Y., M.S. and S.Z. performed the experiments; D.S., D.B. and M.H. analyzed the data; D.S., D.B., M.H. and P.E.J. wrote the article. All authors read and approved the final article.
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Figure legends

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**Figure 2.** Segregation of *viridis-k* mutants. Ten-day-old seedlings germinated from a spike of heterozygous plants of *vir-k.23* and *vir-k.170*. Mutants homozygous for the recessive lethal *viridis-k* loss-of-function alleles are yellow due to deficiency in chlorophyll biosynthesis. The white bar is 5 cm.

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**Figure 4.** Amino-acid sequence alignment of VirK orthologs. Identical residues and residues with similar physical properties are boxed in black and grey, respectively. Cysteine residues participating in iron-sulfur cluster binding are indicated by asterisks. The Ala-118-Thr substitution caused by the *vir-k.170* mutation is indicated above the barley sequence. Sequences were aligned with Clustal Omega (Sievers et al., 2011). Chloroplast
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**Figure 5.** Gene structure of barley *Viridis-k*.

(A) The analyzed region of the *Viridis-k* gene consisting of seven exons; E1-E7. The *vir-k.170* point mutation is located in E4. (B) The *vir-k.23* mutation was mapped by PCR using genomic DNA as template. Gene regions that could and could not be amplified are indicated by filled or dashed lines, respectively. The mapping and linkage analyses suggested a large chromosomal rearrangement interrupting the gene in the region between the two red vertical bars. (C and D) RT-PCR amplicons separated by agarose gel electrophoresis and stained with Midori Green. First strand cDNA synthesis was performed with total RNA isolated from *vir-k.23*, *vir-k.170* and the cultivar Bonus, followed by end-point PCR. (C) Amplification of a 237 bp cDNA fragment using a forward primer located in exon 1 and a reverse primer located in exon 2. (D) Amplification of a 152 bp cDNA fragment using a forward primer located in exon 5 and a reverse primer located in exon 7. The 152 bp fragment could not be amplified from *vir-k.23*.

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Barley *Viridis*-k Links an Evolutionary Conserved C-Type Ferredoxin to Chlorophyll Biosynthesis

David Stuart, Malin Sandström, Helmy M. Youssef, Shakhira Zakhrabekova, Poul Erik Jensen, David Bollivar, Mats Hansson

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