Kim *et al. Applied Biological Chemistry* (2023) 66:81
https://doi.org/10.1186/s13765-023-00838-7

KSABC
The Korean Society for Applied Biological Chemistry

INVITED REVIEW

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Pleiotropic properties of GOLDEN2-LIKE transcription factors for crop improvement

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Abstract

Crop improvement can be affected by enhancing the efficiency of photosynthesis-associated bioprocesses such as chlorophyll biosynthesis, chloroplast biogenesis, the functioning of photosystems including light-harvesting complexes, and carbon fixation. To achieve this, the GOLDEN2-LIKE (GLK) transcription factors represent promising targets since they play a positive role for greening traits in diverse plants. To scrutinize the pleiotropic impact of GLKs, we summarized all phenotypic traits reported in functional studies that used transgenic approaches to lose or gain gene functions. Additionally, we also discussed altered plant phenotypes with respect to their physiological–biochemical aspects and environmental stress responses. From these results, we conclude that GLKs consistently increase chlorophyll biosynthesis, enhance chloroplast division, and increase photosynthetic rate. They individually influence other traits including yield, phytochemical accumulation, and biotic and abiotic stress resistance. Collectively, GLKs have potential as key regulators to effect increases in overall agricultural quality across plant species. This suggests that they may be among the most promising target genes for future agro-biotechnology applications.

Keywords Carotenoids, Chlorophyll, Chloroplast, GLK, Photosynthesis, Yield

Introduction

To increase crop yield and quality, the greening trait during plant vegetative stages, has been used to enhance chlorophyll biosynthesis, chloroplast development, photosynthetic efficiency, and resistance against biotic and abiotic stresses. More precisely, direct regulation of target genes involved in greening-related mechanisms is the primary strategy. To achieve this, several transcription factors (TFs) have received significant attention as key regulators. These include two positive GOLDEN2-LIKE (GLK) and ELONGATED HYPOCOTYL5 (HY5) TFs as well as negative TFs such as PHYTOCHROME INTERACTING FACTORS (PIFs). GLKs are also known as master regulators of chloroplast biogenesis by positively coordinating the expression of photosynthesis-associated nuclear genes (PhANGs) in many plants, including Arabidopsis (*Arabidopsis thaliana*), moss (*Physcomitrella patens*), rice (*Oryza sativa*), tomato (*Solanum lycopersicum*), hybrid birch (*Betula platyphylla*×*B. pendula*), peanut (*Arachis hypogaea*), and liverwort (*Marchantia polymorpha*) [1–9]. From these results, the influence of GLKs on the transcriptional levels of PhANGs appears to be widespread among plant species.

Historically, in 1912, the name "GOLDEN (G)" originated from the golden color phenotype of maize (*Zea mays*) mutant that behaved recessively according to simple Mendelian inheritance [10]. This first mutant was named as *golden1* (*g1*) following discovery of a second mutant, *golden2* (*g2*), which also showed a golden plant color phenotype in maize [11]. Seventy years later, *G2* was cloned as a novel transcriptional regulator for chloroplast development and its hypothesized function was to maintain C_4 development due to bundle sheath defects found in the leaves of maize *g2* mutants [12]. Three years later, the name "GOLDEN 2-LIKE (GLK)" was

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given to the new member of the same family in maize, *ZmGLK1*, which was then distinguished from *G2*, as well as two rice orthologs, *OsGLK1* and *OsGLK2*, which both showed higher amino acid sequence relatedness to *ZmGLK1*/*ZmG1* than *G2* [13]. This finding supported the notion that GLKs function redundantly in mesophyll cells for common C_3 photosynthesis but *G2* acts in a bundle sheath cell-specific manner for C_4 photosynthesis.

GLKs, which belong to a subgroup of the GARP (GOLDEN2 in *Zea mays*; ARR-B, in *Arabidopsis thaliana*; Psr1 in *Chlamydomonas reinhardtii*) superfamily, show strong structural conservation. Each consists of a nuclear localization signal, a DNA-binding domain (DBD), AREAEAA hexapeptide, a proline-rich domain, and a GLK/C-terminal (GCT) box [13, 14]. The DBD is a MYB-related B domain (~60 amino acids in length) that conserved in GARP superfamily with three α-helices. The consensus sequence of the AREAEAA hexapeptide motif, which immediately follows the third helix of the B domain confers specific characteristics to GLKs that distinguish them from other GAPR members [1, 15]. In addition, the GCT is also present in other members of the GARP family including PSEUDO RESPONSE REGULATOR2 proteins [13, 16]. Interestingly, ancestral GLK orthologs emerged in some green algae species in which the GCT box was absent. Later, GLKs with a GCT box diversified with one, two, or more copies in most land plants from bryophytes to angiosperms, with the exception of the obligate parasite *Sapria himalayana*, which completely lacks photosynthetic ability [9, 15, 17]. Taken together, these evidences strongly support the key function of GLKs for chloroplast development.

To date, evidence supports the notion that GLKs coordinate internal transcriptional networks to ultimately incite chloroplast development by sensing and reacting to signals from diverse sources, including light, plastid retrograde signals, phytohormones, senescence, and biotic and abiotic stresses [15, 17]. Moreover, the direct regulation of gene expression by interaction by GLKs with the promoters of target genes has been confirmed for chlorophyll biosynthesis and photosynthetic apparatus in Arabidopsis, peanut, and hybrid birch [4, 7, 8, 18]. Furthermore, other cases of direct promoter binding by GLKs has been reported for *Camellia sinensis* with respect to two catechin biosynthetic genes and in Arabidopsis for three B-BOX domain proteins related to flowering time as well as four ABA-signaling genes [19–21]. Meanwhile, miscellaneous protein partners that are known to interact with GLKs have been reported for a wide range of traits in various plants: tomato for senescence and fruit development, peach (*Prunus persica*) for auxin signaling, apple (*Malus domestica*) for chloroplast development, peanut for water stress, Arabidopsis for cell death, brassinosteroid signaling, cotyledon opening, and photosynthesis, and radish (*Raphanus sativus*) for chlorophyll biosynthesis [8, 22–31]. The fact that GLKs are involved in diverse molecular mechanisms supports the idea that they may improve overall photosynthetic performance and other important traits such as development, growth, and environmental response.

In this review, we briefly summarize phenotypic influences of GLKs on diverse plants as identified by transgenic knockout, functional complementation, or overexpression approaches. In doing so we focus on the physiological–biochemical aspects of plants. Moreover, we also consider the effects of GLKs on resistance to biotic and abiotic stress following loss- or gain-of-function experiments. This review will advance our understanding of the useful features of GLKs and unveil their great potentials with pleiotropic impacts for crop improvement. Finally, we suggest prospects for their application to substantial crop improvement projects.

Greening trait improvement

Plants are autotrophic organisms and therefore depend on light to sustain life functions. Photosynthesis requires a chlorophyll pigment and a chloroplast organelle to perform photosynthesis at scale when exposed to light. Nevertheless, their regulation may occur by specific regulatory mechanisms within the plant body. Here, we categorized recent research of the GLK's impacts on plant greening traits into three major factors, chlorophyll biosynthesis, chloroplast development, and photosynthesis.

Chlorophyll biosynthesis

Chlorophylls, which include both chlorophyll *a* and chlorophyll *b*, are photosynthetic pigments that cause plants to appear green. In general, chlorophyll levels are the strongest indicator of plant greening traits. Therefore, chlorophyll content has been considered as a major characteristic during reverse genetic studies of *GLKs*.

Since a paler yellow–green ("golden") color was observed in a maize *g2* mutant plant [1, 11, 12, 18, 32], loss-of-functional studies involving diverse plant *GLKs* have been performed using diverse biotechnologies including transposon insertion, T-DNA insertion, RNA interference, CRISPR-Cas9-based genome editing, homologous recombination, virus-induced gene silencing, and gene cosuppression (Table 1). Knock-out (KO) phenotypes of *GLKs* were first reported in Arabidopsis, which has two *GLK* genes, and these were found to display a normal green leaf color in both single KO mutants but a pale green in a double KO mutant [1]. Correlated with these color traits, chlorophyll content was significantly decreased in the leaves, siliques, and seedlings in the double mutant but not in either single mutant,

Table 1 Physiological and biochemical phenotypes of loss-of-function GLK mutants and functional complementation results in a double KO Arabidopsis GLK mutant

GLK mutant and gene		Transgenesis			Phenotype in target organ					References
Name (Accesion number)	Plant Source (Scientific name)	Technique (Promoter)	Host plant	Green color trait in plant	Target organ	Chlorophyll content	Chloroplast development	Photosynthesis	Carotenoid content	Flavonoid biosynthesis	
Loss of function											
g2/bundle sheath defective (bsd)1-m1	Maize (*Zea mays* L.)	Transposon insertion	Maize	Paler yellow green (Golden)	Leaf						Jenkins et al. 1926; Hall et al. 1998
atglk1 (At2g20570)	Arabidopsis (*Arabidopsis thaliana* L.)	Transposon insertion	Arabidopsis	Normal green	Leaf						Fitter et al., 2002
atglk2 (At5g44190)		Transposon insertion	Arabidopsis	Normal green with pale green silique	Leaf						Fitter et al., 2002
atglk1atglk2 (At2g20570 At5g44190)		Transposon insertion	Arabidopsis	Pale green	Leaf						Fitter et al., 2002; Waters et al., 2009
atglk1atglk2 (At2g20570 At5g44190)		Transposon insertion	Arabidopsis	Pale green	Siliques						Waters et al., 2008
atglk1atglk2 (At2g20570 At5g44190)		T-DNA insertion	Arabidopsis	Pale green	Leaf						Liu et al., 2018
atglk1 (At2g20570)		T-DNA insertion	Arabidopsis	Normal green	Seedling						Zhao et al., 2021
atglk2 (At5g44190)		T-DNA insertion	Arabidopsis	Normal green	Seedling						Zhao et al., 2021; Liu et al., 2021
atglk1atglk2 (At2g20570 At5g44190)		T-DNA insertion	Arabidopsis	Pale green	Seedling						Zhao et al., 2021
atglk1atglk2 (At2g20570 At5g44190)		T-DNA insertion	Arabidopsis	Pale green	Callus						Sun et al., 2022
Anti-AhGLK1 (KX168636)	Peanut (*Arachis hypogaea* L.)	RNA interference (AhGLK1)	Peanut	Not mentioned	Hairy root						Liu et al., 2020
yellow-green leaf (yl) with a 40-kb deletion containing *BpGLK1*	Hybrid birch (*Betula platyphylla* × *Betula*	T-DNA insertion	Hybrid Birch	Pale green	Leaf						Gang et al., 2019
BpGLK1-reduced expression (Bpev01.c0167.g0013.m0001)	Hybrid birch (*Betula platyphylla* × *Betula*	RNA interference (35S)	Hybrid Birch	Pale green	Leaf						Gang et al., 2019
mpglk	Liverwort (*Marchantia polymorpha* L.)	CRISPR/Cas9	Liverwort	Pale green	Gemmae						Yelina et al., 2023
osglk1 (Os06g24070)	Rice (*Oryza sativa* L.)	RNA interference (Rice Actin1)	Rice	Normal green	Leaf						Wang et al., 2013
osglk2 (Os01g13740)		T-DNA insertion	Rice	Normal green	Leaf						Wang et al., 2013
osglk1osglk2 (Os06g24070 Os01g13740)		RNA interference (Rice Actin1)	Rice	Pale green	Leaf						Wang et al., 2013
PabGLK RNAi	Hybrid poplar (*Populus alba* × *Populus*	RNA interference (35S)	Hybrid Poplar	Pale green	Leaf						Li et al., 2021
ppglk1 (AY741684)	Moss (*Physcomitrella patens* subsp.)	Homologous recombination	Moss	Normal green	Protonema and gametophores						Yasumura et al., 2005
ppglk2 (AY741685)		Homologous recombination	Moss	Normal green	Protonema and gametophores						Yasumura et al., 2005
ppglk1ppglk2 (AY741684 AY741685)		Homologous recombination	Moss	Pale green	Protonema and gametophores						Yasumura et al., 2005
TRV-PpGLK1 (Prupe.3G127700)	Peach (*Prunus persica* L.)	Virus-Induced gene silencing	Peach	Pale green	Leaf and unripe fruit						Chen et al., 2018
CS 35S:SlGLK1 (JQ316460)	Tomato (*Solanum lycopersicum* L.)	Cosuppression (35S)	Tomato	Pale green	Leaf and unripe fruit						Nguyen et al., 2014
slglk2 (SOLYC10G008160)		CRISPR/Cas9	Tomato	Not mentioned	Unripe fruit						Niu et al., 2022
Functional complementation											
AtGLK1 (At2g20570)	Arabidopsis (*Arabidopsis thaliana* L.)	Overexpression (35S)	*Atglk1Atglk2*	Fully rescued	Leaf						Yasumura et al., 2005
PpGLK1 (AY741684)	Moss (*Physcomitrella patens* subsp.)	Overexpression (35S)	*Atglk1Atglk2*	Partially rescued	Leaf						Yasumura et al., 2005
AtGLK1 (At2g20570)	Arabidopsis (*Arabidopsis thaliana* L.)	Overexpression (35S)	*Atglk1Atglk2*	Fully complemented	Leaf and siliques						Waters et al., 2008; Waters et al. 2009
AtGLK2 (At5g44190)		Overexpression (35S)	*Atglk1Atglk2*	Fully complemented	Leaf and siliques						Waters et al., 2008; Waters et al. 2009
AtGLK1 (At2g20570)		Overexpression (FDH)	*Atglk1Atglk2*	No complemented	Leaf						Waters et al., 2008
AtGLK2 (At5g44190)		Overexpression (FDH)	*Atglk1Atglk2*	No complemented	Leaf						Waters et al., 2008
AtGLK1 (At2g20570)		Overexpression (RbcS)	*Atglk1Atglk2*	Fully complemented	Leaf						Waters et al., 2008
AtGLK2 (At5g44190)		Overexpression (RbcS)	*Atglk1Atglk2*	Fully complemented	Leaf						Waters et al., 2008
AtGLK1 (At2g20570)		Overexpression (AtSUC2)	*Atglk1Atglk2*	Partially complemented	Leaf						Waters et al., 2008
AtGLK2 (At5g44190)		Overexpression (AtSUC2)	*Atglk1Atglk2*	Partially complemented	Leaf						Waters et al., 2008
AhGLK1 (KX168636)	Peanut (*Arachis hypogaea* L.)	Overexpression (35S)	*Atglk1Atglk2*	Fully restored	Leaf						Liu et al., 2018
BpGLK1 (Bpev01.c0167.g0013.m0001)	Hybrid birch (*Betula platyphylla* × *Betula*	Overexpression (35S)	*yellow-green leaf (yl)*	Fully rescued	Leaf						Gang et al., 2019
MdGLK1 (MDP0000260794)	Apple (*Malus domestica* L.)	Overexpression (35S)	*Atglk1Atglk2*	Fully complemented	Seedling and silique						An et al., 2019
PpGLK1 (Prupe.3G127700)	Peach (*Prunus persica* L.)	Overexpression (35S)	*Atglk1Atglk2*	Fully complemented	Leaf and silique						Chen et al., 2018
						Decreased	Not changed		Complemented	Not examined	

T-DNA: transfer DNA, CRISPR/Cas9: clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9, 35S: cauliflower mosaic virus 35S promoter, FDH: Arabidopsis epidermal FIDDLEHEAD promoter, RbcS: tomato rubisco small subunit 3b promoter, AtSUC: Arabidopsis companion cell–specific expression promoter

thereby suggesting the functional redundancy of two GLKs in most photosynthetic tissues [4, 18, 32–34]. In cases of moss and rice, both of which have two *GLK* copies, greening-related plant colors and chlorophyll levels of wild type and single mutant plants were indistinguishable, but the double mutant was paler green and showed decreased chlorophyll content; this finding supports the same functional redundancy in green tissues such as moss protonema, gametophores, and rice leaves [2, 35]. Furthermore, single knock-down (KD) and KO mutations have been used to decrease the chlorophyll content of the target organs of several plants, including the hairy roots of peanut, the leaves of hybrid birch and hybrid poplar, the gemmae of liverwort, and the leaves and unripe fruits of peach and tomato. In each case, paler green color traits were observed in *GLK*-suppressed transgenic plants than the wild type, which suggests that GLKs have a substantial impact on chlorophyll biosynthesis [6–9, 23, 29, 36].

To assess the role of *GLKs* from different plants (i.e., Arabidopsis, moss, peanut, apple, and peach),

complementation assays have been performed with the pale green Arabidopsis double mutant (*atglk1atglk2*). For these tests, the chlorophyll content was measured to determine whether other GLKs could functionally rescue native GLK function (Table 1). First, the chlorophyll deficiency of an *atglk1atglk2* leaves was found to be complemented fully by *AtGLK1* and partially by *PpGLK1* (moss), suggesting that there is both functional conservation and divergence between these two plant GLKs [2]. Individual complementation of *AtGLK1* and *AtGLK2* also showed complete rescue of the double mutant phenotype in leaves and siliques overexpressed using the *35S* promoter [4, 32]. In this study, changing the promoter to facilitate differential tissue-specific GLK expression resulted in different degrees of complementation associated with different leaf phenotypes. For example, full rescue was achieved using the photosynthetic cell-specific *RbcS* promoter, partial complementation was achieved using the phloem-specific *AtSUC2* promoter, and no complementation was achieved using the epidermis-specific *FDH* promoter. Moreover, phenotypic rescues of an *atglk1atglk2* were successfully performed using overexpression of heterogeneous *GLKs* under the *35S* promoter; this was observed in leaves by *AhGLK1* (peanut), in leaves and seedlings by *PpGLK1* (peach), and in seedlings and siliques by *MdGLK1* (apple) [18, 23, 24]. Additionally, a T-DNA yellow-green leaf (*yl*) mutant of hybrid birch was successfully rescued with respect to its greening color and chlorophyll level via constitutive overexpression of *BpGLK1* (birch) [7]. Taken together, these results confirm that GLKs can affect chlorophyll content when expressed in photosynthetic cells, and that this mainly occurs in a cell-autonomous manner.

Next, to reveal the effects of GLKs on plant physiological characteristics, overexpression studies have used *GLKs* from diverse plants (Table 2). First, ectopic

Table 2 Summary of altered physiological and biochemical phenotypes following overexpression of GLKs

GLK mutant and gene		Transgenesis			Phenotype in target organ							References
Name (Accesion number)	Plant Source (Scientific name)	Technique (Promoter)	Host plant	Green color trait in plant	Target organ	Chlorophyll content	Chloroplast development	Photosynthesis	Yield	Carotenoid content	Flavonoid biosynthesis	
Gain of function												
AtGLK1 (At2g20570)	Arabidopsis (*Arabidopsis thaliana* L.)	Overexpression (35S)	Tomato (*uniform ripening (u)/u* variety)	Normal green	Immature fruit	■	■			■		Powel et al., 2012
AtGLK2 (At5g44190)		Overexpression (35S)	Tomato (*u/u* variety)	Normal green	Immature fruit	■	■			■		Powel et al., 2012
AtGLK1 (At2g20570)		Overexpression (RbcS)	Tomato (*u/u* variety)	Normal green	Immature fruit	■						Powel et al., 2012
AtGLK2 (At5g44190)		Overexpression (RbcS)	Tomato (*u/u* variety)	Normal green	Immature fruit	■						Powel et al., 2012
AtGLK1 (At2g20570)		Overexpression (LTP)	Tomato (*u/u* variety)	Normal green	Immature fruit	■						Powel et al., 2012
AtGLK2 (At5g44190)		Overexpression (LTP)	Tomato (*u/u* variety)	Normal green	Immature fruit	■						Powel et al., 2012
AtGLK1 (At2g20570)		Overexpression (PDS)	Tomato (*u/u* variety)	Normal green	Immature fruit	□						Powel et al., 2012
AtGLK2 (At5g44190)		Overexpression (PDS)	Tomato (*u/u* variety)	Normal green	Immature fruit	□						Powel et al., 2012
AtGLK1 (At2g20570)		Overexpression (35S)	Arabidopsis	Slightly darker green	Root	■	■	■		■		Kobayashi et al., 2013
AtGLK2 (At5g44190)		Overexpression (35S)	Arabidopsis	Slightly darker green	Root	■	■	■		■		Kobayashi et al., 2013
AtGLK1 (At2g20570)		Overexpression (35S)	Arabidopsis	Slightly darker green	Seedling						■	Zhao et al., 2021
AtGLK2 (At5g44190)		Overexpression (35S)	Arabidopsis	Slightly darker green	Seedling	■	■				■	Liu et al., 2021
AtGLK1 (At2g20570)		Overexpression (35S)	Arabidopsis	Slightly darker green	Callus					■		Sun et al., 2022
AtGLK2 (At5g44190)		Overexpression (35S)	Arabidopsis	Slightly darker green	Callus					■		Sun et al., 2022
AchGLK (Ach17g471351.2)	Kiwifruit (*Actinidia chinensis*)	Overexpression (35S)	Tomato	Normal green	Mature green fruit	■	■			■		Li et al., 2018
AhGLK1 (KX168636)	Peanut (*Arachis hypogaea* L.)	Overexpression (35S)	Peanut	Not mentioned	Hairy root	■						Liu et al., 2020
BpGLK1 (Bpev01.c0167.g0013.m0001)	Hybrid birch (*Betula platyphylla* × *Betula*	Overexpression (35S)	Hybrid Birch	Slightly darker green	Leaf	■	□					Gang et al., 2019
CsGLK1 (MZ093621)	Tea plant (*Camellia sinensis* L.)	Overexpression (35S)	Tomato	Dark green	Leaf and fruit	■	■			■	■	Wang et al., 2022
CsGLK2 (MZ093620)		Overexpression (35S)	Tomato	Dark green	Leaf and fruit	■	■			■	■	Wang et al., 2022
MpGLK	Liverwort (*Marchantia polymorpha* L.)	Overexpression (MpUBE2)	Liverwort	Dark green	Gemmae	■						Yelina et al., 2023
OsGLK1 (AK098909)	Rice (*Oryza sativa* L.)	Overexpression (RiceFOX)	Rice	Normal green	Root and callus	■	■	■				Nakamura et al., 2009
OsGLK1 (AAK50393)		Overexpression (GluB-1)	Rice	Not mentioned	Endosperm				■	■		Li et al., 2022
PabGLK5	Hybrid poplar (*Populus alba* × *Populus*	Overexpression (35S)	Hybrid Poplar	Slightly darker green	Leaf	■		□		■		Li et al., 2021
PpGLK1 (Prupe.3G127700)	Peach (*Prunus persica* L.)	Overexpression (35S)	Tomato	Not mentioned	Unripe fruit	■	■					Chen et al., 2018
SlGLK1 (JQ316460)	Tomato (*Solanum lycopersicum* L.)	Overexpression (35S)	Tomato (*U/U* & *u/u* variety)	Normal green	Immature & mature fruit	■	■			■		Nguyen et al., 2014
SlGLK2 (JQ316459)		Overexpression (35S)	Tomato (*U/U* & *u/u* variety)	Normal green	Immature & mature fruit	■	■			■		Nguyen et al., 2014
SlGLK2 (SOLYC10G008160)		Overexpression (35S)	Tomato	Not mentioned	Unripe fruit	■						Niu et al., 2022
ZmGLK1(ZmG1) (AF318580)	Maize (*Zea mays* L.)	Overexpression (ZmUbi)	Rice	Not mentioned	Leaf	■		■	■	■		Li et al., 2020
ZmGLK2(ZmG2) (AF318579)		Overexpression (ZmUbi)	Rice	Not mentioned	Leaf	■		■	■	■		Li et al., 2020
ZmGLK1(ZmG1) (GRMZM2G026833)		Overexpression (ZmUbi)	Rice	Dark green	Leaf and floret	■	■	■	□			Yeh et al., 2022
ZmGLK2(ZmG2) (GRMZM2G087804)		Overexpression (35S)	Rice	Dark green	Leaf and floret	■	■	■	■			Yeh et al., 2022
ZmGLK1(ZmG1) (GRMZM2G026833)		Overexpression (ZmG1)	Rice	Dark green	Leaf and floret	■	■	■	■			Yeh et al., 2022
ZmGLK2(ZmG2) (GRMZM2G087804)		Overexpression (ZmG2)	Rice	Dark green	Leaf and floret	■	■	■	■			Yeh et al., 2022
ZmGLK1(ZmG1)ZmGLK2(ZmG2) (GRMZM2G026833 GRMZM2G087804)		Overexpression (ZmG1 and ZmG2)	Rice	Dark green	Leaf and floret	■	■	■	■			Yeh et al., 2022

■ Increased □ Not changed Not examined

35S: cauliflower mosaic virus 35S promoter, RbcS: tomato rubisco small subunit 3b promoter, LTP: Arabidopsis lipid transfer protein promoter, PDS: tomato phytoene desaturase promoter, MpUBE2: liverwort ubiquitin-conjugating enzyme E2 promoter, RiceFOX: rice full-length cDNA overexpresser system, GluB-1: rice endosperm-specific glutelin promoter, ZmUbi: maize ubiquitin promoter, ZmG1: maize ZmG1 promoter, ZmG2: maize ZmG2 promoter

expression of *AtGLK1* or *AtGLK2* was performed using four different promoters in a *uniform ripening* (*u*)/*u* tomato variety that exhibits a light green fruit phenotype. Here, a defect in *U* encoding *SlGLK2* resulted in a normal green color for all transgenic tomato plants [5]. Interestingly, in unripe fruits, promoters expressed before ripening—including *35S*, *RbcS*, and *LTP*—resulted in increased chlorophyll levels and a dark green color. However, a *PDS* promoter expressed later during fruit development showed no change. This confirmed that the influence of *GLKs* is apparent only while greening processes are operating. In addition, *AtGLK1* or *AtGLK2* have been constitutively overexpressed in Arabidopsis using the *35S* promoter in multiple studies [33, 34, 37, 38]. In these studies, transgenic Arabidopsis plants exhibited slightly darker green color throughout the plant and showed increased chlorophyll content in examined target organs, including roots and seedlings.

Meanwhile, ectopic overexpression of native *GLKs* has been performed in diverse plant systems. A rice FOX (i.e., a full-length cDNA overexpressor)-mutant designed to activate *OsGLK1* showed a good correlation between greening levels and chlorophyll content in calli, roots, and young seedlings while appearing a normal green color in photosynthetic organs [3]. Additionally, overexpression of two tomato genes, *SlGLK1* and *SlGLK2*, increased the chlorophyll content of dark green immature fruits present on normal green tomato plants [6, 29]. Furthermore, overexpression studies of native GLK genes in hybrid birch (*BpGLK1*), peanut (*AhGLK1*), hybrid poplar (*PabGLK5*), and liverwort (*MpGLK*) all resulted in enhanced chlorophyll levels in target organs, including leaves, hairy roots, leaves, and gemmae, respectively [7–9, 36]. In addition, four GLK genes of peach (*PpGLK1*), kiwifruit (*AchGLK1*), and tea plant (*CsGLK1* and *CsGLK2*) have been constitutively overexpressed using the *35S* promoter in tomato host plants. All transgenic fruits exhibited a darker green color and increased chlorophyll content in unripe stages [23, 39]. Moreover, two tea genes were found to be able to increase chlorophyll levels even in leaves [20]. In cereal crops, similar results were found for two maize GLK genes that were individually overexpressed in rice plants under constitutive promoters such as maize *Ubi* (*ZmUbi*) and *35S*, as well as both individually and together under an endogenous promoter (i.e., *ZmG1* and *ZmG2*, respectively) [40, 41]. For example, all transgenic rice plants showed an increase in chlorophyll content along with a darker green color in their leaves and florets. This evidence strongly supports the potential of maize *GLKs* to improve the greening traits of rice, which may result in higher productivity. As mentioned so far, *GLK* overexpression generally leads to increased chlorophyll content when expressed at the time and place where the photosynthetic mechanism operates; this can be coordinated by using an appropriate promoter. These results clearly indicate that the greatest impact of *GLKs* may be on chlorophyll biosynthesis.

Chloroplast development

Chloroplasts are plant-specific organelles that perform photosynthesis using thylakoids, a novel structure equipped with photosynthetic apparatus as well as pigments such as chlorophylls and carotenoids [42]. Since *GLKs* have been found to regulate chloroplast development in Arabidopsis [1, 32], the biogenesis and development of chloroplasts has been considered as a key trait in loss-of-functional studies of *GLKs* (Table 1). In Arabidopsis leaves, the disordered chloroplast thylakoid ultrastructure of KO mutants was first observed chloroplast phenotype in photosynthetic mesophyll or bundle sheath cells. Here, the authors demonstrated functional redundancy between *AtGLK1* and *AtGlLK2*, both which were less abundant in the thylakoids of a double mutant. However, this was not observed in either single mutant [1, 32]. In the double mutant, the chloroplast number per cell was normal but the size was reduced. Notably, the authors also identified rudimentary thylakoid lamellae as shown on the chloroplasts of the maize *g2* mutant [33]. Later, a single KO mutant, *atglk2*, showed a slightly smaller chloroplast size, lower staking degree, and lower abundance of thylakoid components in Arabidopsis seedlings [34]. Furthermore, much reduced thylakoid-membrane networks and grana stacking in the leaf chloroplasts were reported in T-DNA insertional KO and RNAi-mediated KD mutants of *BpGLK1* in hybrid birch plants [7]. A CRISPR/Cas9-mediated KO mutant (*MpGLK*) in liverwort gemmae showed slightly increased chloroplast number but significantly decreased chloroplast size and scarce thylakoid stacking, suggesting overall inhibition of chloroplast development [9]. Similarly, in moss and rice, both of which have two GLK genes, a double mutant but neither single mutant showed impairment of granal formation in the protonema and gametophore of moss and rudimentary thylakoid development in the leaves of rice [2, 35].

Phenotypes related to chloroplast development have been observed in few cases of functional complementation studies (Table 1). For example, a defective granal phenotype of an *atglk1atglk2* leaves was fully restored by *RbcS::AtGLK1* expression and was partially restored by *AtSUC2::AtGLK2* expression [32]. This suggests GLKs can act cell-autonomously in photosynthetic cells but also act non-cell-autonomously to a limited level of non-photosynthetic phloem companion cells to induce chloroplast development in adjacent mesophyll cells. The same

atglk1atglk2 mutant was completely rescued to wild type levels as measured by the number of granal thylakoids in leaves and siliques by *35S::PpGLK1* expression [23]. In addition, the hybrid birch *y* mutant, which shows a much reduced thylakoid-membrane network was fully rescued by *35S::BpGLK1* expression, showing a well-developed chloroplast with closely stacked grana [7]. As summarized in Table 1, all phenotypic results from loss of function and functional complementation studies suggest that GLKs play a leading role in inducing chloroplast development in photosynthetic tissues and show functional redundancy in cases where two copies are present.

Overexpression studies of *GLKs* have been performed in tomato, Arabidopsis, hybrid birch, and rice (Table 2). All ectopic expressions of GLK genes in tomato were constitutively expressed using the *35S* promoter in an *u/u* variety for *AtGLK1* and *AtGLK2* and common tomato for *SlGLK1*, *SlGLK2*, *PpGLK1*, *AchGLK*, *CsGLK1*, and *CsGLK2* [5, 6, 20, 23, 39]. Transgenic tomato plants commonly showed enhanced chloroplast development in immature/unripe fruits, with increases in number, size, and density of chloroplasts as well as high accumulation in the grana stacking of thylakoids. Similarly, in Arabidopsis, increased chloroplast number and a highly stacked grana structure was formed by overexpression of either *AtGLK1* or *AtGLK2* in roots or *AtGLK2* in seedlings [34, 37]. Meanwhile, constitutive overexpression of native GLK genes in hybrid birch showed little difference from the wild type in the granal stacks of leaf chloroplasts as well as a slight increase in chlorophyll levels, thereby suggesting a somewhat weak effect of *BpGLK1* relative to other cases [7]. In rice, *OsGLK1*-FOX plants ectopically induced chloroplast biogenesis with well-developed thylakoids membranes and grana structures in calli (under light conditions), coleoptiles, and leaf sheaths of young leaves but in mature plants [3]. In contrast, two maize GLK genes, *ZmG1* and *ZmG2*, were found to enhance chloroplast development, causing increased size, number, and significantly enhanced stacking of thylakoid membranes per chloroplast unit area in rice plants [41]. This finding confirmed that—unlike *OsGLK1*—both maize genes can promote chloroplast development even in mature rice plants. Thus, these results show that GLKs are key regulators of chloroplast biogenesis as well as chlorophyll biosynthesis.

Photosynthesis

Photosynthesis is a comprehensive biochemical reaction that takes place in chloroplasts. These organelles are equipped with photosynthetic apparatus, including photosystems and photosynthetic pigments such as chlorophyll [43]. Photosynthetic efficiency has traditionally been considered as a major feature for increasing crop yield. To assess whether GLKs may be useful for this purpose, in what follows we consider plant phenotypes related to photosynthetic efficiency.

As summarized in Table 1, the effect of GLKs on photosynthesis following loss of function was first observed in an Arabidopsis double mutant, *atglk1atglk2*. These plants showed a lower electron transport rate and PSII photochemical yield in the light. These phenotypes were restored comparable to the wild type levels when *AhGLK1* was complementally overexpressed [18]. In hybrid birch, a defect in *BpGLK1* was confirmed to affect photosynthesis in the *yl* mutant. This defect resulted in a significant decrease in net photosynthetic rate (*Pn*), stomatal conductance, transpiration rate, and initial fluorescence, but showed similar values with respect to maximal quantum efficiency of PSII (*Fv/Fm*), photochemical quantum yield of PSII (*ΦPSII*), and photochemical quenching. This result suggests that *BpGLK1*-KO can cause impairment in photosynthetic capacity but does not influence photosynthetic efficiency. Meanwhile, in hybrid poplar, the *PaGLK* RNAi lines have been found to show significantly higher *Pn* without changes in *Fv/Fm* relative to the WT [36]. This indicates that photosynthetic efficiency was not affected by suppression of *PaGLK*. Furthermore, in liverwort the photosynthetic apparatus was found to remain functional without changes in *Fv/Fm* in the gemmae of the gene-edited *mpglk* mutant despite a significant reduction in chlorophyll as well as the appearance of smaller chloroplasts with an altered ultrastructure [9]. Overall, these findings suggest that the influence of GLKs on photosynthesis more strongly determines the structural formation and maintenance of the photosynthetic apparatus than the efficiency itself.

The effects of GLKs on photosynthesis have been observed in numerous overexpression studies (Table 2). In Arabidopsis, ectopic expression of either *AtGLK* or *AtGLK2* improved phototropic activity, the photoautotrophic growth of roots, and caused an increase in CO_2 fixation but not in leaves [37]. However, the *Fv/Fm* levels of these mutants were decreased in roots despite being unchanged in leaves. This finding suggests that the imbalance in the photosynthetic machinery decreases the efficiency of light utilization in the root chloroplasts, which have lower photochemical efficiency than the leaf chloroplasts. In another study, the overexpression of native *PaGLK5* in hybrid poplar showed a significant reduction in *Pn* without changes in *Fv/Fm* relative to the WT [36]. The discrepancy between the decrease in *Pn* and the increase in chlorophyll content indicated that *PaGLK5* does not directly affect photosynthetic efficiency. In rice, *OsGLK1*-FOX, which corresponds to the overexpression of *OsGLK1*, exhibited higher photosynthetic activity in unusually green calli [3]. This suggests that ectopic expression

of native *OsGLK1* enables photosynthesis in non-green (ivory-yellow) cells but is not able to increase greening traits including photosynthetic activity in the mature stages of photosynthetic parts of rice plants. However, individual constitutive overexpression of two maize *GLK* genes in field-grown plants led to improved light-harvesting efficiency via photosystem II with an accompanying increase in values such as stomatal conductance, intercellular CO_2 concentration, *nP*, *ΦPSII*, and *Fv/Fm* [40]. These results were further reproduced using two constitutive promoters (i.e., *ZmUbi* and *35S*) and using two endogenous maize promoters (i.e., *ZmG1* and *ZmG2*) in rice plants, respectively [41]. All transgenic rice plants—either *ZmG1*, *ZmG2*, or *ZmG1/ZmG2*—had higher photosynthetic rates and higher *nP*, stomatal conductance, mesophyll conductance, and light use efficiency in mature flag leaves of 4-month-old stages compared to wild type rice. Accordingly, it was proposed that these two maize *GLK* genes could synergistically improve the photosynthetic performance of rice even when under the control of native promoters.

Yield increase

As global food demand rapidly grows, yield increases are becoming more and more important. Over the past few decades, crop biotechnologists have tried to increase agricultural yield through technological advancements involving molecular biology and plant transformation. In maize, the contribution of genetic improvement was estimated at only 13% compared to 39% derived from agronomic improvement [44].

In Table 2, data related to the yield potential of GLKs is summarized. Rice is a major cereal crop along with corn and wheat and is consumed daily by about 50% of the global population [45]. Two studies reported evidence of yield increases in rice following ectopic overexpression of two maize *GLKs* [41]. One study showed a 30–40% increase in both vegetative biomass and grain yield. This was accompanied by increases in several agronomic traits such as panicle length, panicle weight, and seed number per panicle when a constitutive promoter was used [40]. This finding verified that these increases may be caused by enhanced photosynthetic capacity in field-grown transgenic plants but was not affected by the levels of hormones such as auxin, cytokinin, and gibberellin due to their statistical difference relative to the WT. The other study showed clear positive effects of maize GLKs on grain yields in transgenic rice plants that overexpressed either *ZmG1* or *ZmG2* (or both) using constitutive promoters (i.e., *ZmUbi* or *35S*) or the maize *ZmG1* and *ZmG2* promoters [41]. Most rice plants showed increases in shoot biomass and grain yield traits including total panicle number and total grain weight per plant. In particular, the highest grain yields occurred when both maize *GLKs* were simultaneously expressed under endogenous promoters, and resulted in a 70% increase in grain yield. However, one rice plant overexpressing *ZmG1* under the *ZmUbi* promoter produced smaller seeds without showing an apparent yield increase. These findings prompt us to suggest caution; very high levels of expression using strong constitutive promoters may lead to negative effects on seed development. However, this finding did not agree with those of a previous study, which showed that overexpression of either *ZmG1* or *ZmG2* using the same *ZmUbi* promoter resulted in similarly high grain yields (i.e., best performance increases of 56% and 118% relative to the wild type) during a single season in Hainan. This study was performed using a *japonica*-type Kitaake cultivar but the latter study used a *japonica*-type TNG67 cultivar. This difference suggests the possibility that different results may be obtained depending on the rice host variety [40, 41]. Meanwhile, endosperm-specific overexpression of *OsGLK1* has been found to increase grain yield by approximately 20% as well as increase the values of several components including filled grain per panicle, the seed-setting rate, and grain yield per plant in the conventional rice variety ZH11 [46]. However, this mutant also showed deterioration in rice grain quality, showing seriously increased chalkiness due to spherical, loosely packed, and irregularly polyhedron-shaped grains.

Phytochemical effects

Phytochemicals refer to organic compounds that are naturally produced by plants. They confer self-defense functions against disease and environmental change and supply important health benefits for many heterotrophic organisms. Phytochemicals mainly include terpenoids such as carotenoids, phytosterols, and saponins, as well as polyphenols such as anthocyanins and other flavonoids [47]. Of these, the levels of carotenoids and flavonoids have been reported to be influenced by GLKs. Here, we comprehensively describe the contributions of GLKs on phytochemical content, focusing on the target organs of diverse plants when examined through reverse genetic approaches that examine loss or gain of GLK functions.

Carotenoid biosynthesis

Carotenoids are natural pigments that belong to the terpenoid group. In plants, they perform photosynthesis along with chlorophylls and participate in various biological processes as photo-protectants, antioxidants, precursors of plant hormones. Carotenoids also play roles as color attractants in plants, since they are naturally colored and have diverse color hues (e.g., yellow, orange, and red). This also makes them suitable for use in the production

of food and animal fodder. Moreover, diverse carotenoids such as β-carotene, zeaxanthin, astaxanthin, and capsanthin have received attention as major metabolites involved in plant biofortification, since they provide nutritionally relevant provitamin A components. Moreover, carotenoids can also provide health-promoting ingredients such as antioxidants and other free-radical scavengers [48, 49].

The effects of GLKs on carotenoid content have been reported in many plants, including hybrid birch, Arabidopsis, hybrid poplar, tomato, and rice. As shown in Table 1, decreased carotenoid levels following functional suppression of *GLKs* was first demonstrated in two hybrid birch mutants, *yl* and the *BpGLK1*-reduced expression line [7]. The former was fully rescued by constitutive overexpression of native *BpGLK1* in the leaves of hybrid birch plants (*C-yl*). Similarly, a decrease in carotenoid content was observed in the leaves of the *PaGLK* RNAi-hybrid poplar mutant and in the calli of the Arabidopsis *atglk1atglk2* mutant [36, 38]. Taken together, these results reveal a positive correlation between GLK expression and carotenoid levels and an especially close relationship with chlorophyll levels in photosynthetic leaves.

As shown in Fig. 1 and Table 2, the effects of GLK overexpression on carotenoid content were first reported in the *u*/*u* (*slglk2*) tomato variety via constitutive ectopic expression of either *AtGLK1* or *AtGLK2* using the *35S* promoter [5, 20]. Both genes individually increased lycopene content by 10–60% in ripe fruit, supporting the notion that higher *GLK* expression during early stages could contribute to higher carotenoid content in the later stages of fruit development. In this study, the general selection of *u* for uniform fruit ripeness was determined to be an inadvertent compromise by a tomato breeding program for ripe fruit quality in exchange for

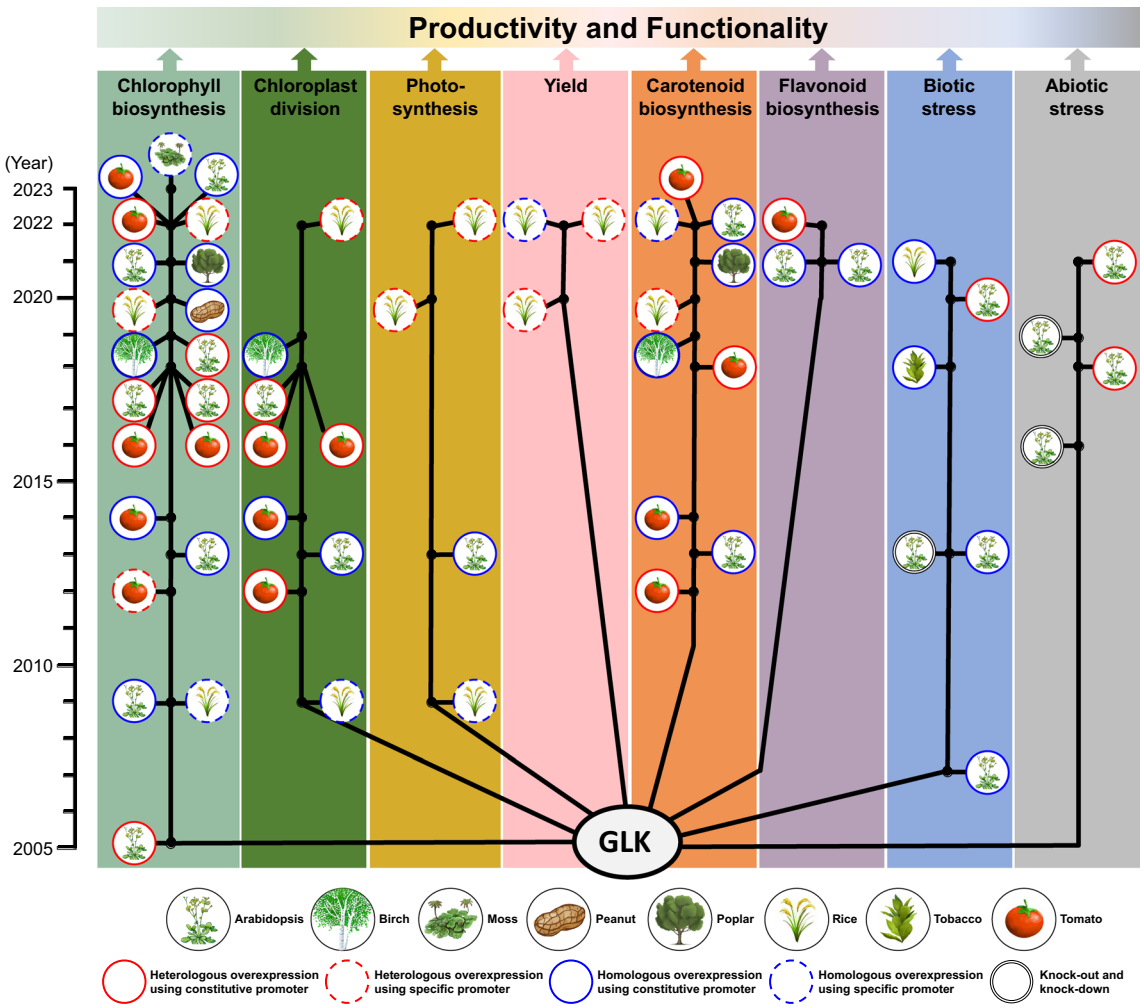

Fig. 1 Positive effects of GLKs on various plant species. The vertical axis is the publication year, and the horizontal axis is displayed separately by trait types to improve crops. Picture source: Arabidopsis, Peanut, Birch, Poplar: craiyon.com; Moss: The image of Moss is from TogoTV (© 2016 DBCLS TogoTV, CC-BY-4.0 https://creativecommons.org/licenses/by/4.0/deed.ja.)

more desirable carotenoid traits. The same Arabidopsis genes were constitutively overexpressed in Arabidopsis to increase carotenoid levels in non-photosynthetic roots, seed-derived calli, and etiolated seedlings [37, 38]. In this study, the molecular mechanism responsible for the direct effects of Arabidopsis GLKs on carotenoid accumulation was hypothesized as follows: GLKs form nuclear condensates with G-BOX BINDING FACTOR which then binds to the *PSY* promoter region, thereby resulting transcriptional upregulation of PSY [38]. In tomato, several GLK genes, *SlGLK1*, *SlGLK2*, *PpGLK1*, *AchGLK*, *CsGLK1*, and *CsGLK2*, were individually overexpressed using the same *35S* promoter [6, 20, 23, 39]. All transgenic tomato plants exhibited enhanced carotenoid and chlorophyll levels in green fruits. This overexpression ultimately resulted in higher total carotenoid content in ripe red fruits, suggesting that the unripe green fruit stage is source of photosynthate, in contrast to the ripe fruit stages. In addition, the homologous overexpression of two native tomato genes, *SlGLK1* and *SlGLK2*, increased β-carotene and lutein content more than lycopene content in the skin of red fruit [6]. Moreover, heterologous overexpression of tea genes, *CsGLK1* and *CsGLK2*, increased the carotenoid content of leaves [20].

In cereal crops such as rice, leaf carotenoids were significantly increased (along with chlorophyll levels) when two maize GLKs were constitutively overexpressed [40]. Grain carotenoid levels were also enhanced relative to a wild type ZH variety following endosperm-specific overexpression of *OsGLK1* (G) [46]. The GLK impact of carotenoid levels was confirmed by co-expression with three carotenogenic genes (i.e., *tHMG1*, *ZmPSY1*, and *PaCrtI*, which encode the enzymes truncated 3-hydroxy-3-methylglutaryl-CoA reductase, phytoene synthase, and phytoene desaturase, respectively) together (GHPC). This revealed that *OsGLK1* could increase total carotenoid content by nearly threefold relative to HPC rice endosperm. Taken together, introduction of GLKs appears to have a major impact on carotenoid production in both source and sink organs of important crops such as a rice.

Flavonoid biosynthesis

Flavonoids, a major class of phenolic compounds, comprises several subclasses such as anthocyanins, flavonols, flavones, isoflavones, and flavanones. In plants, they play essential roles in mediating plant responses to biotic and abiotic environmental factors. In addition, in humans they confer health benefits since they scavenge free radicals, thereby contributing protection against broad spectrum of diseases [47]. Of the flavonoids, anthocyanins are water-soluble natural pigments that are responsible for the red, blue, and purple colors found in fruit and floral tissues. Catechins are flavonol-type polyphenols that are accumulated in tea plants [20, 33, 34].

Reduced anthocyanin levels following loss-of-function mutations have been reported only in Arabidopsis (Table 1). Moreover, the degree of reduction differed among Arabidopsis mutants: the lowest was observed in the double mutant, the next lowest in the *atglk2* single mutant, and no effect was observed in the *atglk1* single mutant [33]. Furthermore, decreased anthocyanin content following the disruption of *AtGLK2* was reconfirmed in Arabidopsis seedlings [34]. In these studies, increased anthocyanin levels following gain-of-function manipulations have also been reported in Arabidopsis (Table 2). Independent overexpression of either *AtGLK1* or *AtGLK2* resulted in a significant increase in anthocyanin accumulation in seedlings [33, 34]. Interestingly, both studies also suggested putative molecular mechanisms by which AtGLK1 may regulate anthocyanin biosynthesis. The former suggested that AtGLK1 positively regulates sucrose-induced anthocyanin biosynthesis via action upstream of the MYB-LIKE2 (MYBL2) TF, which is a key negative regulator of anthocyanin biosynthesis [33]. The latter study proposed that AtGLK2 positively regulates anthocyanin biosynthesis via ELONGATED HYPOCOTYL 5-mediated light signaling in Arabidopsis as well as by antagonistic function between AtGLK2 and AtMYBL2 [34]. Meanwhile, overexpression of two tea *GLKs* (i.e., *CsGLK1* or *CsGLK2*) increased total flavonoid content—especially catechin by 4.49- and 4.05-fold in tomato fruits, respectively [20]. This study also proposed that *CsGLKs* are positively regulators of light-regulated catechin accumulation in tea plants by activating R2R3-type CsMYB5b. Finally, they speculated that GLKs may have great potential for simultaneous enhancement of the accumulation of catechins and carotenoids in the fruits of horticultural crops such as tomato.

Stress resistance

Plants face diverse biotic and abiotic stresses and in order to survive, have evolved mechanisms of detecting and responding to stresses on the physiological, biochemical, and molecular levels. To consider whether GLKs have resistance to either biotic or abiotic stresses, we compiled all possible phenotypes reported when *GLKs* were suppressed or overexpressed using transgenic approaches (Table 3).

Biotic stress

Plants are constantly exposed to various pathogens such as fungi, bacteria, and viruses which reduce in crop productivity [50]. Therefore, enhancing pathogen resistance is an important target trait for many types of agriculture. In the previous section, we described the

Table 3 Summary of the influence on biotic and abiotic stress resistance following suppression and overexpression of GLKs in diverse plants

GLK gene		Transgenesis			Resistance to stress		Reference
Name (Accession number)	Plant Source (Scientific name)	Technique (Promoter)	Host plant	Resistant target	Biotic	Abiotic	
Loss of function							
atglk1 (At2g20570)	Arabidopsis (*Arabidopsis thaliana* L.)	T-DNA insertion	Arabidopsis	*Cucumber mosaic virus*			Han et al., 2016
atglk2 (At5g44190)		T-DNA insertion	Arabidopsis	*Cucumber mosaic virus*			Han et al., 2016
atglk1atglk2 (At2g20570 At5g44190)		T-DNA insertion	Arabidopsis	*Cucumber mosaic virus*			Han et al., 2016
atglk1atglk2 (At2g20570 At5g44190)		T-DNA insertion	Arabidopsis	*Hyaloperonospora arabidopsidis Noco2*			Murmu et al., 2014
atglk1atglk2 (At2g20570 At5g44190)		T-DNA insertion	Arabidopsis	*Botrytis cinerea*			Murmu et al., 2014
atglk1 (At2g20570)		Repression by SRDX fusion	Arabidopsis	Ozone			Nagatoshi et al., 2016
atglk2 (At5g44190)		Repression by SRDX fusion	Arabidopsis	Ozone			Nagatoshi et al., 2016
atglk1atglk2 (At2g20570 At5g44190)		T-DNA insertion	Arabidopsis	Osmotic and dehydration			Ahmad et al., 2019
ghglk1 (Gh_D01G0183)	Cotton (*Gossypium Tomentosum x Gossypium hirsutum*)	Virus-induced gene silencing	Cotton	Cold and drought			Liu et al., 2021
osglk1 (Os06g24070)	Rice (*Oryza sativa* L.)	T-DNA insertion	Rice	Rice black-streaked dwarf virus			Li et al., 2022
Gain of function							
AtGLK1 (At2g20570)	Arabidopsis (*Arabidopsis thaliana* L.)	Overexpression (35S)	Arabidopsis	*Fusarium graminearum*			Savitch et al., 2007
AtGLK1 (At2g20570)		Overexpression (35S)	Arabidopsis	*Hyaloperonospora arabidopsidis Noco2*			Murmu et al., 2014
AtGLK1 (At2g20570)		Overexpression (35S)	Arabidopsis	*Botrytis cinerea*			Murmu et al., 2014
AtGLK1 (At2g20570)		Overexpression (35S)	Arabidopsis	Ozone			Nagatoshi et al., 2016
AtGLK2 (At5g44190)		Overexpression (35S)	Arabidopsis	Ozone			Nagatoshi et al., 2016
AtGLK1 (At2g20570)		Overexpression (35S)	Arabidopsis	Osmotic and dehydration			Ahmad et al., 2019
AtGLK2 (At5g44190)		Overexpression (35S)	Arabidopsis	Osmotic and dehydration			Ahmad et al., 2019
AhGLK1 (KX168636)	Peanut (*Arachis hypogaea* L.)	Overexpression (35S)	Arabidopsis	Drought			Liu et al., 2018
AhGLK1b (MK952147)		Overexpression (35S)	Arabidopsis	*Sclerotinia sclerotiorum*			Ali et al., 2020
AhGLK1b (MK952147)		Overexpression (35S)	Arabidopsis	*Pseudomonas syringae pv. tomato DC3000*			Ali et al., 2020
GhGLK1 (Gh_D01G0183)	Cotton (*Gossypium Tomentosum x Gossypium hirsutum*)	Overexpression (35S)	Arabidopsis	Cold and drought			Liu et al., 2021
NbGLK1 (Niben101Scf06721g00011.1)	Tobacco (*Nicotiana benthamiana* L.)	Overexpression (35S)	Tobacco	*Potato virus X*			Townsend et al., 2018
OsGLK1 (Os06g24070)	Rice (*Oryza sativa* L.)	Overexpression (Ubi)	Rice	*Rice black-streaked dwarf virus*			Li et al., 2022

Increased Decreased Not applicable

T-DNA: transfer DNA, SRDX: the ERF-associated amphiphilic repression (EAR) motif repression domain, 35S: cauliflower mosaic virus 35S promoter, Ubi: ubiquitin promoter

positive effects of GLKs on plant growth, agricultural yield, and phytochemical. Here, we will consider the biotechnological applicability of GLKs on disease resistance (Table 3).

The first role GLKs play in plant defense was demonstrated in Arabidopsis by constitutive overexpression of *AtGLK1*. This resulted in resistance against to *Fusarium graminearum*, a fungal pathogen responsible for major losses in cereal crops such as wheat and maize, where it causes *Fusarium head blight* and *gibberella ear mold*, respectively [51]. It was suggested that overexpression of *AtGLK1* may reprogram gene expression networks to upregulate defense-related genes as a resistance mechanism. Further elucidation of the GLK roles in plant defense involved examining two different pathogens, *Hyaloperonospora arabidopsidis* (*Hpa*) Noco2 (a biotrophic oomycete) and necrotrophic *Botrytis cinerea* (a necrotrophic fungus) [52]. Interestingly, when taken together these results showed conflicting patterns of resistance associated with GLKs; namely, *atglk1atglk2* plants exhibited higher resistance against *Hpa* Noco2 while *35::AtGLK1* plants showed heightened resistance against *B. cinerea*. Ultimately, *AtGLKs* were confirmed to act on jasmonic acid (JA)-dependent susceptibility to *Hpa* Noco2 and JA-independent immunity to *B. cinerea*. Another dual resistance study involving two biotic pathogens, *Sclerotinia sclerotiorum* (a necrotrophic fungal pathogen) and *Pseudomonas syringae pv. tomato DC3000* (a phytopathogenic bacterium), was reported in Arabidopsis plants that showed constitutive overexpression of peanut *AhGLK1b* [53]. Here, the suggested function of *AhGLK1b* in plant disease resistance involved the upregulation of defense-related genes including a multidrug and toxin extrusion efflux protein, PR10, and Phox/Bem 1, which is involved in multiple disease resistance.

Furthermore, the involvement of GLKs in plant resistance to viruses was first investigated in Arabidopsis in

response to infection by *Cucumber mosaic virus* (*CMV*) [54]. Researchers found that *atglk1atglk2* plants were more susceptible than *Atglk1* and *Atglk2* to CMV and suffered more serious damage. This included higher oxidative damage, more compromised PSII photochemistry, and more reactive oxygen species accumulation [54]. Taken together, these findings suggested that *AtGLK1* and *AtGLK2* might play redundant roles in *CMV* resistance in Arabidopsis. A second case was reported in tobacco plants that overexpressed tobacco *NbGLK1* and were then subjected to *Potato virus X* (*PVX*) infection [55]. This study found that *NbGLK1* can act as an immune activating protein to *reduce susceptibility to PVX* by activation (i.e., de-repression) via activated *Retinal homeobox gene 1*, an intracellular immune receptor, following virus perception. Rice is also damaged by viruses, the most destructive of which is *rice black-streaked dwarf virus* (*RBSDV*) [56]. Quantitative trait loci (QTL) analysis using the *RBSDV*-resistant Wuke variety explored three genes associated with *RBSDV* resistance. Interestingly, *OsGLK1* was identified as a major resistance QTL. This finding was reinforced by an empirical test of whether resistance against *RBSDV* was supported by *OsGLK1*; this involved both KD and the stable overexpression of *OsGLK1* in rice plants.

Abiotic stress

Due to their sessile life history, plants experience diverse environmental stresses including ultraviolet radiation, drought, cold, heat, and salinity [50]. A variety of abiotic stresses affect the growth and development of plants and ultimately cause reduced agricultural productivity. To further explore the influence of GLKs on abiotic stress resistance, we reviewed the results of phenotypic changes (Table 3).

High concentrations of ozone (O_3), the most phytotoxic air pollutant in the troposphere, is known to cause oxidative stress in plants and is associated with crop losses. One study in Arabidopsis showed that a strong tolerance to ozone was associated with a closed-stomata phenotype caused by suppression of either *AtGLK1* or *AtGLK2* via fusion of the SRDX, the ERF-associated amphiphilic repression motif repression domain [57]. In contrast, the overexpression of either *AtGLK1* or *AtGLK2* caused plants to exhibit higher sensitivity to ozone when plants showed an open-stomata phenotype. Taken together, these findings suggest that *AtGLK*s may play a positive role in regulating the expression of genes related to K + ion channels. It has also been proposed that AtGLKs may be effective tools for conferring resistance to air pollutants by controlling stomatal movement. For instance, another study of Arabidopsis *atglk1atglk2* mutant plants revealed osmotic stress resistance during seedling development accompanied by abscisic acid (ABA)-hypersensitivity [19]. Exactly contrary to this, the overexpression of these two genes showed hypersensitivity to osmotic and salt stresses and an ABA-hyposensitive phenotype, respectively. This evidence suggests that AtGLKs are associated with resistance to osmotic and dehydration stress in Arabidopsis.

Next, two cases have identified a relationship between GLKs and drought resistance in crops that are greatly affected by drought. In peanut, overexpression of native *AhGLK1* increased survival rates during recovery from drought in Arabidopsis [18]. AhGLK1 enables post-drought recovery by stimulating chlorophyll biosynthesis and photosynthesis via the unregulated expression of *AhPORA*. Moreover, cotton crops are also exposed to harsh environments during cultivation. Virus-induced silencing of *GhGLK1* in cotton resulted in plants that were more vulnerable to drought and cold stress, whereas its overexpression in Arabidopsis showed greater adaptability following drought and cold treatments than the wild type [58]. Taken together, these findings suggest that *GhGLK1* might be a key candidate gene for the simultaneously enhancement of cold and drought stress tolerance.

Current potential and future prospects for crop improvement

In this review, we evaluated the impact of GLKs regarding overall greening traits, yield, phytochemical accumulation, and stress resistance. We did so by examining different forms of plant transformation research, including gene knocking-out by insertion of transposons and T-DNA, CRISPR/Cas9-mediated genome editing, knocking-down by RNA interference, homologous recombination, virus-induction, and repressor domain fusion, and overexpression using diverse promoters. Through these studies, we identified positive effects of GLKs on chlorophyll biosynthesis, chloroplast development, photosynthesis, and carotenoid biosynthesis, as well as significant effects in particular cases on flavonoid biosynthesis and resistance to specific pathogens and forms of stress. As shown in Fig. 1, all positive results were listed chronologically. Moreover, our analysis shows that functional studies of GLKs have mainly focused on Arabidopsis for most traits. However, GLK studies have consistently attempted to improve agricultural traits in a diverse range of crops, including birch and poplar (wood), peanut (a legume), rice (a cereal), tobacco, and tomato (a vegetable). While chlorophyll and chloroplast traits were the primary consideration in most plant studies, photosynthesis and yield were a primary interest for rice studies, and differences in the abundance of two functional metabolites—i.e., carotenoids and flavonoids were identified in tomato fruits.

From the functional standpoint, several notable features are evident. First, there is evidence of function redundancy between *GLK* copies that rarely change greening traits in single KO mutants and show functional differences depending on promoter (e.g., *35S*, *Ubi*, *RbcS*, *AtSUC2*, *LTP*, *MpTBE2*, *ZmG1*, and *ZmG2*, but not *FDH* and *PDS*). Thus, tests requiring photosynthetic cell expression may not be effective in non-photosynthetic tissues such as roots, calli, dark-grown seedlings, ripe fruits, and seeds (Tables 1 and 2). Second, other finding strongly suggest that GLKs enhance photosynthetic performance (i.e., result in increased chlorophyll and carotenoid biosynthesis, increased chloroplast biogenesis, and more efficient photosynthetic machinery) in photosynthetic tissues. Moreover, enhanced photosynthates then contribute to improvements in yield and functionality via increased carotenoid and flavonoid content in both non-photosynthetic sink organs and photosynthetic source organs.

Furthermore, the influence of GLKs on biotic stress resistance was positively correlated for three fungi, including cereal pathogen *F. graminearum* as well as two necrotrophs *B. cinerea* and *S. sclerotiorum*, along with the phytopathogenic bacterium *P. syringae pv. tomato DC3000.* In Arabidopsis, *AtGLK1* overexpression plant was susceptible against a biotrophic oomycete *Hpa* Noco2 compare to *atglk1atglk2* [51, 52, 54]. GLK overexpression also conferred positive resistance against three pathogenic viruses, including *CMV* (i.e., *AtGLKs* in Arabidopsis), *PVX* (i.e., *NbGLK1* in tobacco), and *RBSDV* (i.e., *OsGLK1* in rice) [53, 55, 56]. Moreover, abiotic stress resistance was increased for drought and cold stress when *AhGLK1* and *GhGLK1* were individually overexpressed and for higher ozone concentration and osmotic stress when *AtGLKs* were knocked-out in Arabidopsis [18, 19, 57, 58]. Taken together, these results demonstrate that GLKs have great potential for improving resistance against diverse forms of stress (Fig. 1).

Given the above considerations, this review also shows that using an appropriate promoter is essential for applying GLKs to crop improvement. A late ripening-specific promoter (*PDS*) failed to generate a carotenoid increase in ripe tomato fruits [5]. However, a strong constitutive promoter (*ZmUbi*) for *ZmG1*- not *ZmG2*-overexpression led to negative impacts on seed development and endosperm-specific overexpression. Finally, *GluB-1* can cause deteriorating grain quality due to increased chalkiness in rice [41, 46].

We currently face a food crisis caused by rapid population growth and global climate change. Consequently, the discovery of major genes influential enough to increase agricultural productivity may be the most important goal in plant biology. The use of GLKs to improve agricultural biotechnology has great potential for the improvement of numerous crops and may facilitate the sustainable survival of humanity via food security.

Acknowledgements
Not applicable.

Author contributions
Conceptualization: SK, HC, SH; data curation: SK; writing—original draft preparation: SK, HC; tables and figure production: SK, TY, DG; writing—review and editing: SK, HC, SH; supervision: SK, HC, SH; Funding acquisition: SH All authors have read and agreed to the published version of the manuscript.

Funding
This work was supported by a grant from the Research Programs (NRF-2021R1A2C2012227 to S-HH) through the National Research Foundation funded by the Ministry of Science and ICT, South Korea and also a grant from the New Plant Breed Technology Program (RS-2022-RD009998 to S-HH) funded by the Rural Development Administration, South Korea.

Availability of data and materials
Not applicable.

Declarations

Competing interests
The authors have no competing interests to declare that are relevant to the content of this article.

Received: 6 October 2023 Accepted: 2 November 2023
Published online: 23 November 2023

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