Phytochrome-regulated Gene Expression

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Abstract

Identification of all genes involved in the phytochrome (phy)-mediated responses of plants to their light environment is an important goal in providing an overall understanding of light-regulated growth and development. This article highlights and integrates the central findings of two recent comprehensive studies in *Arabidopsis* that have identified the genome-wide set of phy-regulated genes that respond rapidly to red-light signals upon first exposure of dark-grown seedlings, and have tested the functional relevance to normal seedling photomorphogenesis of an initial subset of these genes. The data: (a) reveal considerable complexity in the channeling of the light signals through the different phy-family members (phyA to phyE) to responsive genes; (b) identify a diversity of transcription-factor-encoding genes as major early, if not primary, targets of phy signaling, and, therefore, as potentially important regulators in the transcriptional-network hierarchy; and (c) identify auxin-related genes as the dominant class among rapidly-regulated, hormone-related genes. However, reverse-genetic functional profiling of a selected subset of these genes reveals that only a limited fraction are necessary for optimal phy-induced seedling deetiolation.

Key words: expression profiling; phytochrome signaling; transcriptional networks; microarrays; reverse genetics; functional profiling; transcription factors; auxin-related genes.


Plants use informational light signals from the environment to modulate their growth and development in accord with prevailing conditions throughout the life cycle. For this purpose they have evolved small families of sensory photoreceptors that monitor various parameters of the impinging radiation, including color, intensity, direction and periodicity (Schäfer and Nagy 2006). Among these receptors, the phytochrome (phy) family, consisting of five members, phyA through phyE in *Arabidopsis*, are responsible for tracking the red (R) and far-red (FR) regions of the spectrum (Tu and Lagarias 2005). These molecules are activated by absorption of photons that switch them from the inactive conformer, Pr, to the active conformer, Pfr. This activation triggers rapid translocation of the photoreceptor from the cytoplasm into the nucleus where it initiates a cascade of changes in gene expression that drive the observable changes in growth and development referred to as photomorphogenesis (Chen et al. 2004; Nagatani 2004; Quail 2006).

Although a considerable number of early studies documented the expression patterns of a limited set of phy-regulated genes using conventional molecular methodologies (Tobin and Kehoe 1994; Terzaghi and Cashmore 1995; Kuno and Furuya 2000), global analysis of photoregulated genes has only been possible relatively recently since the introduction of microarray technology (Lipshutz et al. 1999). Initial microarray-based studies aimed at defining the spectrum of photoresponsive genes regulated by phyA and phyB were performed with arrays of only about 6–8 000 genes ("8K" microarrays), representing 25–30% of the *Arabidopsis* genome (Ma et al. 2001, 2002;
Tepperman et al. 2001, 2004; Wang et al. 2002), whereas more recent studies have been done with arrays that provide closer to full-genome coverage (\(\geq 80\%\)) (“22K” microarrays) (Monte et al. 2004; Jiao et al. 2005; Ma et al. 2005; Tepperman et al. 2006). One group of these studies measured presumptive end-point, steady-state transcript profiles after prolonged (5- to 6-day), continuous FR (FRc) or continuous R (Rc) irradiation (Jiao et al. 2005; Ma et al. 2001, 2002, 2005), whereas the other group followed the time-course of changes in expression over the first 24 hr of irradiation (Tepperman et al. 2001, 2004), or focused specifically on genes responding to the light signal within 1 hr of initial exposure of seedlings to Rc, in order to enhance the probability of identifying those genes most likely to be direct targets of the phy signaling pathway (Monte et al. 2004; Tepperman et al. 2006). These investigations have defined the molecular phenotype of wild-type seedlings during deetiolation in Arabidopsis in response to the Rc and FRc light signals, and have begun to dissect the photosensory functions of phyA and phyB in this process using phyA and phyB null mutants.

The early 8K-microarray data verified that phyA is exclusively responsible for regulation of the genes that respond to FRc signals in these seedlings (Ma et al. 2001; Tepperman et al. 2001). However, surprisingly, the majority of Rc-responsive genes on these arrays were found to remain relatively strongly responsive to this light signal in the phyB null mutant, suggesting that one or more of the remaining phy family members is predominantly responsible for perception and transduction of this signal (Ma et al. 2001; Tepperman et al. 2004). Subsequent studies with the “22K” microarrays defined the genome-scale molecular phenotypes of wild-type seedlings in response to Rc and FRc light (Monte et al. 2004; Jiao et al. 2005), but did not address the responsiveness of the phyA and phyB mutants genome-wide, leaving unanswered the question of which phy family member(s) is predominantly responsible for Rc-regulated gene expression during deetiolation. This question has been addressed recently by defining the genome-scale expression profiles of phyA and phyB monogenic, and phyAphyB double mutants in response to Rc using the Affymetrix ATH1 ("22K") microarray (Tepperman et al. 2006).

Despite the power of microarray-based expression profiling for identifying most or all of the phy-regulated genes in the genome, these data alone do not establish the functional relevance of these genes to phy-controlled morphogenetic responses. This question can be addressed by examining the effects of systematic targeted reverse-genetic disruption of these photosensitive genes on the photomorphogenic responses of the mutated plants. One such recent study has utilized this functional profiling strategy to begin to examine this question (Khanna et al. 2006).

This article summarizes highlights both from the abovementioned recent microarray analyses of phy-regulated gene expression (focused on those genes responding early to the inductive light signal) (Tepperman et al. 2006), and from the functional profiling study (Khanna et al. 2006).

### Microarray-based Expression Profiling

#### Definition of Rc-regulated, phy-dependent early-response genes

To identify early-response genes and define the potential roles of phyA and phyB in their regulation, genome-scale, light-induced changes in expression were analyzed, using Affymetrix ATH1 microarrays, in wild-type, phyA, phyB and phyAphyB mutant seedlings after initial transfer of dark-grown seedlings to Rc for 1 hr (Tepperman et al. 2006). A flowchart summarizing the sequential steps in this analysis is presented in Figure 1A, together with scatter plots displaying the expression levels of the relevant genes at each step, in the 1-hr Rc-irradiated seedlings compared to the unirradiated, dark-controls (Figure 1B–G). This analysis identified a total of 1,459 genes that were either induced or repressed in the wild type by 1 hr Rc, in a statistically significant fashion (Figure 1A). The distribution of the expression levels of these genes is depicted by the red dots in the relevant scatter plot (Figure 1C). Of these, 251 genes were induced (206) or repressed (45) 2-fold or more by the Rc signal relative to the dark-controls (Figure 1A), and were therefore defined as quantitatively robustly- to moderately-responsive to this signal. These genes are denoted by the green dots in the relevant scatter plot (Figure 1D).

Further analysis of the expression of these genes in the phyA, phyB and phyAphyB mutants identified those genes whose response to 1-hr Rc differed in a statistically significant fashion from that of the wild type (Figure 1A). For these genes, the magnitude of the contribution of each phy, alone or in combination, to the Rc-responsiveness of each gene was defined as the Fold-Induction Ratio (FIR) for induced genes, or Fold-Repression Ratio (FRR) for repressed genes (Monte et al. 2004; Tepperman et al. 2004, 2006). This parameter quantitatively measures the magnitude of the change in expression (Fold-Induction (FI) or Fold-Repression (FR)) induced by the Rc treatment in the wild type compared to each phy-mutant genotype, expressed as a ratio (wild-type/phy mutant). A ratio of 1.0 signifies no detectable contribution of that phy photoreceptor species to the light-induced response, independently of whether the underlying response to light is intrinsically large or small for that gene. Deviations from 1.0 provide a quantitative measure of the robustness of the contribution of that phy family member to the rapid Rc induced response. Those genes with FIR and FRR values of \(\geq 1.5\) were defined as robustly \(\geq 2.0\) to moderately \(1.5–2.0\) dependent on the relevant phys for Rc-responsiveness (Figure 1A). These genes are denoted by the blue dots in the relevant scatter plot for each genotype,
**Induced genes**

Figure 2A shows the means of the expression levels of all 206 Rc-induced genes for all four genotypes, and Figure 2B displays the fold-induction for each individual gene in each genotype arrayed by descending fold-induction in wild type. The data show that most these genes (>90%) display greater dependence on phyA than phyB for the full, wild-type level of responsiveness to the Rc signal. phyA is necessary for the principal quantitative component of the early-light-responsiveness of most of these Rc-responsive genes. No other phy family member, including phyB, is fully redundant with phyA in Rc signaling to these genes. Conversely, the phyB data, showing that only 9 genes (4%) exhibit robust to moderate (FIR ≥1.5) dependence on phyB (Figure 1A,F), suggest that phyB is not necessary for the normal, maximal Rc-light induction of these genes. On the other hand, 96% of these genes are robustly to moderately reduced in their Rc-responsiveness (FIR≥1.5) in the absence of both photoreceptors in the phyAphyB double mutant (Figure 1 and 2A,B). These data indicate that a significant proportion of these genes do, in fact, display a detectable to substantial dependence on phyB for the residual Rc-responsiveness observed in the phyA mutant. On the other hand, the absence of any statistically significant residual Rc-responsiveness in all but three of the 206 induced genes in the phyAphyB double mutant, indicates that neither phyC, phyD nor phyE have a significant role in this response (data not shown). Taken together these data reveal the striking dominance of phyA in the induction of early-response genes during initial exposure to Rc, with phyB playing only a minor role in this process in the presence of phyA. On the other hand, phyB does contribute significantly to Rc-induced expression in the absence of phyA, indicative of partial redundancy under these conditions (Tepperman et al. 2006).

**Repressed genes**

Figure 3A shows the means of the expression levels of all 45 Rc-repressed genes for all four genotypes, and Figure 3B displays the fold-repression for each individual gene in each genotype, arrayed by descending fold-repression in wild type. Of these 45, only 7% (3 genes) are statistically- and quantitatively robustly- to moderately-dependent on phyA, and only 2% (1 gene) dependent on phyB for this rapid Rc repression (FRR ≥1.5; Figures 1, 3A). These data show that, in contrast to the Rc-induced genes, the majority of the early-response genes that are repressed by Rc are not robustly regulated by phyA during the first 1 hr of Rc, and indicate that neither phyA nor phyB is individually necessary for the near-full, negative Rc-light responsiveness of most of these genes. On the other hand, 64% (29) of these genes are robustly to moderately reduced in their Rc-responsiveness (FRR≥1.5) in the absence

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**Figure 1.** Flow-chart showing the numbers of genes identified at sequential steps in the microarray expression analysis, and scatter plots displaying the expression levels of the relevant genes at each step, in the 1-hr Rc-irradiated seedlings compared to the unirradiated, dark-controls (Tepperman et al. 2006).

(A) The sequential steps involved, first, identification, within the approximately 22 000 genes on the Affymetrix ATH1 microarray, of those genes whose expression changed (either induced or repressed) in statistically significant fashion in wild-type (WT) seedlings in response to 1 hr Rc compared to unirradiated dark-control WT seedlings. Within this set of 1 459, those genes were identified whose expression changed (induced or repressed)≥2-fold in WT seedlings in response to 1 hr Rc (induced or repressed) compared to unirradiated dark-control seedlings. Within this set of 251, those genes were identified that displayed a statistically significant difference in expression (induction or repression) between WT and each of the phy mutant genotypes (phyA, phyB or phyAphyB) in response to 1 hr Rc. Within each of these sets, those genes were identified that displayed a quantitatively robust to moderate dependence on phyA, phyB or phyA plus phyB (combined), as defined by a 1-hr-Rc-induced fold-change (FC; either induction (FI) or repression (FR)), that was 1.5-fold-greater in WT seedlings than in the respective phy mutant genotypes (phyA, phyB or phyAphyB), i.e., a fold-change ratio (FCR) of ≥1.5, where FCR = (FC in WT)/(FC in phyX). Percent values represent the percentage of total induced (206) or repressed (45) genes in each category.

(B–G) Scatter plots displaying the expression levels of the relevant genes at each step, in the 1-hr Rc-irradiated seedlings compared to the unirradiated, dark-controls.

(B) All approximately 22 000 genes (grey dots) on the Affymetrix ATH1 microarray in wild-type (WT) seedlings. Diagonal lines denote 2-fold increase (induced) or decrease (repressed) in expression at 1 hr in Rc compared to dark control.

(C) Early Rc-responsive genes, statistically different in expression between dark control and 1 hr-Rc-irradiated WT seedlings (red dots).

(D) Robustly early Rc-responsive genes, displaying ≥2-fold change in expression at 1 hr Rc in WT seedlings compared to dark control (green dots).

Early Rc-responsive genes displaying quantitatively robust- to moderate-dependence on (E) phyA, (F) phyB, or (G) phyA plus phyB combined for Rc-responsiveness (blue dots). (Modified from Tepperman et al. 2006).
of both photoreceptors in the phyAphyB double mutant (Figures 1, 3), indicating that these genes are partially regulated in redundant fashion by phyA and phyB combined, during the first 1 hr of Rc. However, the majority of the 45 repressed genes display apparent residual responsiveness to the Rc signal in the phyAphyB double mutant (Figure 3A,B). Indeed, statistical analysis indicates that 18 (40%) of these genes display statistically significant Rc-repression compared to the dark control in this mutant. Thus, the evidence suggests that one or more of the remaining three phy family members, phyC, phyD and/or phyE, contribute significantly to the residual Rc-responsive-ness of these genes. Together these data indicate that phyA and phyB act in mutually redundant fashion to partially repress these genes with varying degrees of combined effectiveness, but that in addition, one or more of the phyC/phyD/phyE trio also act partially redundantly, or additively, with the phyA/phyB pair, again to varying degrees, to exert full Rc-imposed repression of expression (Tepperman et al. 2006).

**Functional categories**

The distribution of the 206 Rc-induced genes among broadly-defined functional categories is shown in Figure 4A (Tepperman et al. 2006). The data indicate that putative or established
transcription-factor-encoding genes comprise the largest single class of the currently annotated genes in this induced set (27%), and are 3-fold over-represented relative to their occurrence on the ATH1 microarray and in the Arabidopsis genome (9% of ATH1 GeneChip; http://arabidopsis.med.ohio-state.edu/AtTFDB). This dominance is even more striking within the most strongly-induced subset of these genes where 50% of the 22 most strongly Rc-responsive genes are in this category (data not shown). Putative signaling-component genes are also a prominent class (14% of annotated). A number of genes that have been previously implicated in phy-mediated light responses, including HY5, CCA1, LHY, APRR9, APRR5, HYH, SPA1, PKS1, ELF4, GI and FKF1, as well as numerous additional potential regulators are present in these two categories. Of particular interest is SIGE, a nuclear encoded sigma factor, considered likely to function as a regulatory subunit of plastid-localized RNA polymerase (Allison 2000) and therefore to have a potentially key, and possibly global, role in regulating phy-induced expression of genes in the chloroplast genome. Collectively, the data indicate that these transcription-factor genes are early, if not primary, direct, targets of phy signaling, and, therefore, have the potential to function as regulators of multiple downstream target genes in the phy-regulated transcriptional network.

The distribution of the 45 Rc-repressed genes among functional categories is shown in Figure 4B (Tepperman et al. 2006). Like for the Rc-induced genes, the data indicate that putative or established transcription-factor-encoding genes comprise the largest single class of the currently annotated genes in this set (41%), once again strongly over-represented (more than 4-fold) relative to their occurrence in the Arabidopsis genome (9% of ATH1 GeneChip; http://arabidopsis.med.ohio-state.edu/AtTFDB). These encompass a diversity of classes, including bHLH, zinc-finger and trihelix factor-encoding genes, and the growth factor gene, AtIGRF7(AT5G53660; Kim et al. 2003). However, the most prevalent class is the homeomain-leucine zipper (HD-ZIP) class, including ATHB2(HAT4) and ATHB4, both of which have been previously implicated in phy-regulated responses (Morelli and Ruberti 2002; Salter et al. 2003). Also notable is a high representation of hormone related genes in the repressed set (18% of annotated) compared to the induced set (1.5%). Yet more interesting, is the dominance of these two functional categories in the 18 set of repressed genes identified as partially phyC-, phyD- and/or phyE-dependent, where 60% of the currently annotated genes are classified as transcription-related and 27% as hormone-related (data not shown).

Most striking, however, is the dominance of auxin-related genes within both the induced and repressed hormone-related sets. These genes, which include SAUR(AT4G38840) and GH3-L(AT4G03400) (induced), and GH3-L(AT2G23170), SAUR-AC1 (AT4G13790), AUX/IAA(AT4G32280), IAA19(AT3G15540) and HOOKLESS(HLS)(AT4G37580), (repressed), comprise 78% of the total annotated, hormone-related, early Rc-responsive genes (Tepperman et al. 2006). This observation raises the possibility that these auxin-related genes may be direct targets of phy signaling, perhaps providing a mechanism by which the phy rapidly modulate cell expansion rates in hypocotyls and/or cotyledons via the auxin system in response to Rc.

Particularly pertinent to this suggestion is the behavior of HLS. Li et al. (2004) have shown that the level of the endogenous HLS protein in dark-grown Arabidopsis seedlings declines rapidly (t1/2 ~ 1 hr) upon exposure to light. Evidence is presented that HLS functions to suppress the levels of the auxin response transcription factor, ARF2, and consequently ARF2 levels rise concomitantly with the light-induced decline in HLS levels. The data indicate further that the accumulation of ARF2 leads in turn to the unbending of the apical hook by apparent over-riding of the effects of the existing differential auxin gradient across the hook that otherwise maintains differential cell-expansion rates between concave and convex sides. Interestingly, Li et al. (2004) reported that the light-induced decline in the level of HLS protein supported by the endogenous HLS gene did not occur when expression of this gene was driven by the 35S promoter. These data might suggest that the decline in HLS levels is not the result of direct light-induced degradation of the HLS protein, as appears to happen with PIF3 (Kim et al. 2003; Bauer et al. 2004; Monte et al. 2004; Al-Sady et al. 2006). Instead, we suggest that regulation occurs at the gene expression level. We propose that our data showing Rc-induced repression of HLS gene expression (Tepperman et al. 2006), coupled with the data of Li et al. (2004), are consistent with the proposal that phy-mediated Rc signals rapidly repress HLS gene transcription, which leads to the observed decline of HLS protein levels as a result of the extant constitutive turnover rate, and consequently to hook unbending, as proposed by Li et al. (2004). Thus, HLS may represent the primary target through which phy induces hook unbending.

Functional profiling of phy-regulated early-response genes

To begin to determine whether these early-response, robustly light-regulated genes are functionally relevant to phy signal transduction in the overall, global process of seedling deetiolation, we selected an initial subset of 32 genes, categorized as having potential transcriptional-regulatory or signaling activity, as well as several unannotated sequences, for systematic analysis by reverse-genetic disruption (Khanna et al. 2006). For this purpose we isolated, and/or characterized for photomorphogenic defects, insertional-disruption or other known mutants in these genes, using the well-established reciprocal, concomitant inhibition of hypocotyl cell elongation
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Figure 5. Only a limited fraction of phy-early-response genes are necessary for optimal deetiolation.

Seven lines (22%) display concomitant, reciprocal disruption of normal hypocotyl and cotyledon cell expansion in response to Rc (Class I). Seven lines (22%) display significant, parallel (non-reciprocal), global defects in Rc responsiveness in both hypocotyl and cotyledon cell expansion (Class II); Six lines (19%) show significant enhanced, Rc-imposed inhibition of only hypocotyl cell expansion, with no effect on normal cotyledon cell expansion (Class III); and 12 lines (37%) display no detectable aberrant seedling phenotypes (Class IV). The gene name designation and the corresponding Arabidopsis Genome Initiative (AGI) number for the genes in each class are listed. (Modified from Khanna et al. 2006).
and stimulation of cotyledon cell expansion induced by light signals (see Quail 2002) as diagnostic criteria for defining loci most likely to be involved in central regulatory steps in the overall deetiolation process. The results of this analysis are summarized in Figure 5.

Only 7 of these genes (22%) caused statistically significant, reciprocal, aberrant photoresponsiveness in the two organs, when mutated (Class I), indicative of disruption of normal deetiolation. The remainder either had no effect on seedling light-responsiveness (Class IV), when mutated, or caused light-response aberrations that were either indicative of global defects in general cell expansion processes (Class II), or lacked evidence of clear involvement in central, upstream events specific to phy signaling (Class III). This observed high proportion of phy-regulated early-response genes that appear not to be functionally necessary for the overt, wild-type seedling deetiolation phenotype (Khanna et al. 2006) is consistent with similar investigations in other model systems, such as yeast, where less than 7% of responsive genes were found to be functionally necessary for optimal growth under the imposed stress environment (Giaever et al. 2002). Potential reasons for this observation in Arabidopsis include the possibility that these genes are functionally irrelevant to this process, are functionally redundant, or are only transiently involved in an early function during the initial transition from dark to light, with no observable longer-term impact under these conditions.

Of the 7 early-response loci defined as having functions in the overall deetiolation process, six (PRR9, PIL1, HY5, PKS1, SPA1, ELF4) have been independently identified as being involved in light signaling (Koornneef et al. 1980; Ang et al. 1998; Eriksson et al., 2003; Hoecker et al. 1998, 1999; Fankhauser et al. 1999; Khanna et al. 2003; Lariguet et al. 2003; Salter et al. 2003), whereas the SOUL-1 locus had not been previously identified as being phy-related. While this observation provides independent verification of the earlier results, it suggests that this approach to functional profiling may not necessarily yield a large number of new genes involved in phy-regulated signaling and transcriptional networks. It is also notable that the phenotypes caused by these monogenic mutants, are relatively moderate compared to the most extreme hypersensitive and hyposensitive phenotypes observed for phyB-null mutants and phyB-overexpressors, respectively (Khanna et al. 2006). This observation indicates that although these loci appear to have a level of functional importance in light-induced deetiolation, none is singularly essential sufficiently early in the phy-induced signaling cascade to pleiotropically affect the full development of the deetiolation response. This may not be surprising given that the large number of forward genetic screens that have been performed over the years, may have been expected to have identified the majority of monogenic mutations causing robust visible deetiolation phenotypes. Higher order mutants will need to be tested to examine these loci for possible functional redundancy or other interactions.

Conclusions

Microarray expression profiling has identified a set of about 250 genes that respond relatively rapidly and robustly to the Rc-light signal. About 80% of these genes are induced, and about 20% repressed. This gene-set provides a molecular phenotype that represents the initial changes induced by the signal, and provides insight into the potentially primary signaling and transcriptional networks involved in transducing this signal (Tepperman et al. 2006).

Exploitation of this molecular phenotype has revealed differential channeling of the Rc signal through individual phy family members to these early-response genes during initial exposure of seedlings to light. The data indicate that phyA and phyB combined are responsible for mediating the full extent of Rc-induced expression of 96% of the induced genes. No evidence of substantial involvement of any of the remaining family members, phyC, phyD or phyE, in this process was obtained. Moreover, phyA dominates in transducing the Rc signals to these early-induced genes, with phyB playing a more subsidiary role, apparent only in the absence of phyA. In striking contrast, phyA and phyB have a markedly less dominant role in Rc-imposed repression of early-response gene expression, indicating a significant role for one or more of the other three phys, phyC, phyD and/or phyE, in this response. The data thus reveal a previously unrecognized dichotomy in signaling to induced and repressed genes within the phy family (Tepperman et al. 2006).

Examination of the established or predicted functional roles of the early-response genes indicates that transcription-factor-encoding genes represent the largest single category, for both induced and repressed genes, at a frequency three to four times their prevalence genome-wide. The data indicate that these genes are early, if not primary, direct, targets of phy signaling, and, therefore, have the potential to function as regulators of multiple downstream target genes in the phy-regulated transcriptional network. In addition, hormone regulated genes are strongly represented in the repressed gene-set, with the large majority of these being auxin-related. Intriguingly, however, auxin-related genes are also the majority of induced hormone-related genes (Tepperman et al. 2006). This apparent simultaneous, converse phy regulation (induction and repression) of different components of the auxin pathway might reflect organ-specific responses, such as those observed in hypocotyls and cotyledons, which exhibit concomitant opposite cell-expansion responses to light.

Initial, systematic functional profiling by reverse-genetic disruption of a subset of these early light-responsive genes, to examine their functional relevance to the expression of the
normal, visible, photomorphogenic phenotype, has established that only a relatively low proportion of these genes are individually necessary for optimal seedling deetiolation (Khanna et al. 2006). This finding is consistent with similar, more extensive studies in other systems such as yeast. Apart from possible technical explanations, such as the inadequacy of currently available statistical procedures for identification of biologically relevant changes in gene expression levels by microarray, potential reasons for this phenomenon may include functional redundancy across coordinately regulated, but largely sequence-unrelated factors, such as those identified in these studies.

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References


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