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Coordinate Gene Response to Salt Stress in *Lophopyrum elongatum*[1]

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ABSTRACT

***Lophopyrum elongatum* is a highly salt-tolerant relative of wheat. A previous study showed that the abundance of a number of mRNA species is enhanced or reduced in the roots of the *L. elongatum* × *Triticum aestivum* amphiploid by salt stress. Eleven genes with enhanced expression in the roots of salt-stressed *L. elongatum* plants have been cloned as cDNAs. The clones were used as probes to characterize temporal expression of these genes in roots after initiation of salt (250 mM NaCl) stress. All 11 genes are induced within 2 h after exposure to 250 mM NaCl and reached peak expression after 6 h. The decline of gene expression distinguished two groups, one in which mRNA concentrations returned to basal levels by 24 h and the other in which this occurred between 3 and 7 d. One of the 11 clones was found to be homologous to a multigene family of abscisic acid-induced genes, *rab* and *dhn*, identified in other species. We suggest that the coordinate expression of this large number of genes reflects the existence of a highly specific early response to salt stress. We refer to this response as the "early salt stress response."**

Salt-affected soils are a serious agricultural problem throughout the world. Considerable effort has been made to understand salt tolerance in plants to improve agricultural production under saline conditions. Although a wide range of genetic adaptations to saline conditions has been observed and a number of significant physiological responses have been associated with tolerance, the underlying mechanisms of salt tolerance in plants are still poorly understood.

Several lines of evidence indicate that the physiology of plants is altered when they are exposed to salt stress, which can result in an enhanced ability to tolerate stress. In sorghum, exposure of plants to a low level of salt stress enhanced their ability to tolerate a subsequent exposure to a higher level of salt stress (1). Identical observations were made in bread wheat, *Lophopyrum elongatum* (Host) A. Love ($2n = 2x = 14$) (syn. *Elytrigia elongata* [Host] Nevski, *Agropyrum elongatum* Host) and their amphiploid (J. Dvořák, unpublished results).

In *Mesembryanthemum*, salt stress results in a switch from C_3 to CAM-based photosynthesis (3). At the molecular level, exposure of plants to salt was shown to enhance or reduce abundance of specific proteins (5, 14–16) or specific mRNA species (7, 11, 22). Similar observations were made in cell cultures of tobacco and tomato (17, 23). It seems reasonable to conclude that these changes are not accidental but, rather, reflect specific physiological processes that lead to acclimation of the plant to growth under salt stress.

To study these physiological processes we isolated a large number of cDNA clones from *L. elongatum* that show enhanced expression when the plant is salt stressed (12). These clones were used to measure expression of their corresponding genes in *L. elongatum* after the initiation of salt stress. Changes in protein profiles as seen by two-dimensional gel electrophoresis can be caused either by changes in steady-state level of proteins or by posttranslational modifications of the proteins that change their electrophoretic mobility. Northern blot analysis directly detects changes in steady-state levels of mRNAs of individual genes; thus, the analysis is not confounded by factors that can affect protein measurements.

These experiments were designed to determine whether increased steady-state levels of mRNA for this group of genes were constant for the duration of the stress and, if not, to characterize the dynamics of expression. Previous investigation by in vitro mRNA translation of the gene expression in *L. elongatum* × *Triticum aestivum* amphiploid under a salt stress of 250 mM NaCl showed several changes in specific mRNA levels in roots but failed to show any significant changes in shoots (11). For this reason in the present study of gene expression, we concentrated on roots.

L. elongatum is remarkably tolerant of saline environments (19), has a close phylogenetic relationship to cultivated wheats, and is a potential source of genes for the improvement of wheat. The amphiploid between *L. elongatum* and bread wheat, *T. aestivum* L. ($2n = 6x = 42$), expresses salt tolerance (9). Complete sets of disomic chromosome additions and substitutions from *L. elongatum* have been introduced into wheat, and several *L. elongatum* chromosomes have been shown to impart significant levels of salt tolerance in wheat genetic background (8).

MATERIALS AND METHODS

Isolation of cDNA Clones

Lophopyrum elongatum seed were germinated on vertically supported blotting paper and then grown in solution culture

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for 30 d as described previously (11). Plants were then transferred to fresh solution and after 24 h were stressed with 250 mM NaCl. RNA was isolated from the plant roots after 2, 6, 12, and 24 h and 3 and 7 d of treatment and from nonstressed control plants using the guanidium isothiocyanate method of Cathala et al. (4) with modifications (11). Polyadenylated RNA was isolated by oligo(dT)-cellulose chromatography (18). The construction and enrichment of a cDNA library for clones of stress-induced genes by a formamide phenol-emulsion reassociation method is described in detail elsewhere (12).

Northern Hybridization Analysis

Clones that appeared to be from stress-induced genes were characterized by northern blot analysis. Polyadenylated RNAs isolated from the roots of plants stressed for 2, 6, 12, and 24 h and 3 and 7 d and from nonstressed plants were electrophoretically fractionated and blotted onto Hybond-N nylon membranes (Amersham) (10). Inserts of cDNA clones were labeled with ^{32}P by the random primer method (Amersham) and hybridized with the membranes as described earlier (12). Two cDNA clones from constitutively expressed genes, an actin clone from *Bremia lactucae* and an unidentified clone from *L. elongatum*, were used as control probes. Membranes were autoradiographed with Kodak X-AR5 film. The relative abundances of message on northern blots were measured by scanning with a laser densitometer, and data were normalized for the period of exposure of the autoradiograph and by comparison with the controls. The principal band of each lane was quantified; any signal that tailed below the band was not included in the estimate. In the case of multiple bands in a single lane, each band was quantified individually; this occurred only for *ESI*18.

DNA Sequencing

Both DNA strands of clones were sequenced with double-stranded plasmid as a template using the Sequenase sequencing kit (United States Biochemical Corp.). Sequence similarities among the clones were compared using the DNA/Protein Sequence Analysis System (IBI). The University of Wisconsin GCG program for DNA sequence analysis was used for homology searches in the GenBank data base.

RESULTS

Screening of an enriched cDNA library constructed from polyadenylated RNA isolated 6 h after the initiation of salt stress (250 mM NaCl) resulted in the selection of 28 clones of genes with enhanced expression in the roots of salt-stressed *L. elongatum* (12). Cross-hybridization and sequencing of these clones reduced the population to 11 groups, which did not show more than 55% sequence similarity in pairwise comparisons (12). This lack of homology indicated that they are from 11 different genes or gene families in *L. elongatum*. All clones were partial cDNAs encoding the 3′ end of the transcript; the sizes of the clones and the sizes of the corresponding mRNAs inferred from northern blot hybridization are listed in Table I.

Table I. *cDNA Clones from Salt-Stress-Induced Genes from the Roots of L. elongatum*

Length of cDNA clones from salt stress-induced genes does not include homopolymer tails. Estimates of the lengths of corresponding mRNAs are determined from northern blots and are given in numbers of nucleotides (nt).

Clone	cDNA Clone Length	mRNA Length	Relative Level of mRNA[a]
1 *ESI*2	265	950	10
2 *ESI*3	199	700	25
3 *ESI*4	279	1000	8
4 *ESI*14	334	1400	7
5 *ESI*15	125	1100	1
6 *ESI*18	367	700, 1300, 2100	120
7 *ESI*28	179	1100	7
8 *ESI*32	152	1300	25
9 *ESI*35	248	1200	7
10 *ESI*47	820	1300	5
11 *ESI*48	375	950	1

[a] The relative level of mRNA is a comparison of abundances among the salt stress-induced genes taken at maximum expression on northern blots and quantified by laser densitometer; expression is listed relative to the message species with the lowest abundance, *Esi*48. The relative level of expression of *Esi*18 is based on the density of the 1.3-kb band.

The induction patterns of these genes by salt stress were similar. Northern blot analysis showed low or undetectable levels of mRNAs in the roots of plants grown in the absence of NaCl (Figs. 1 and 2). The message levels increased after 2 h and reached peak expression after 6 h (Fig. 2). Genes corresponding to clones *ESI*15 and *ESI*47 were somewhat exceptional; *Esi*15 reached a peak expression earlier and *Esi*47 reached a peak expression later than the other genes. (*ESI* refers to the clone, *Esi* refers to the gene from which the clone was derived.) The decline of expression defined two subgroups, namely, genes whose message levels showed a precipitous decline after 12 h to approximately nonstressed levels by 24 h (*Esi*2, *Esi*4, *Esi*15, *Esi*28, *Esi*32, *Esi*35, and *Esi*48) and those that showed a more gradual decline during a period of 7 d (*Esi*3, *Esi*14, and *Esi*47). The relative level of expression varied more than 2 orders of magnitude among the 11 clones at the time of maximum expression, as judged by the intensity of their signal on northern blots (Table I, Fig. 2).

Clone *ESI*18 showed similar dynamics of expression but was unique in that it hybridized to three bands on the northern blot. The larger two messages, 2.1 and 1.3 kb, appeared by 2 h, and the 1.1-kb message was first seen at 6 h. The 2.1-kb message was not visible after 6 h; the 1.3- and 0.7-kb messages were clearly visible at 24 h and detectable at 3 d (Fig. 1d). The gene(s) corresponding to *ESI*18 were the most strongly expressed among the 11 stress-induced genes isolated.

Nucleotide sequences of all *ESI* clones were used for a search of sequence homology to previously reported sequences in the GenBank data base. Although nucleotide sequences of 10 of the clones did not show homology to any sequence in the data base, *ESI*18 showed high sequence

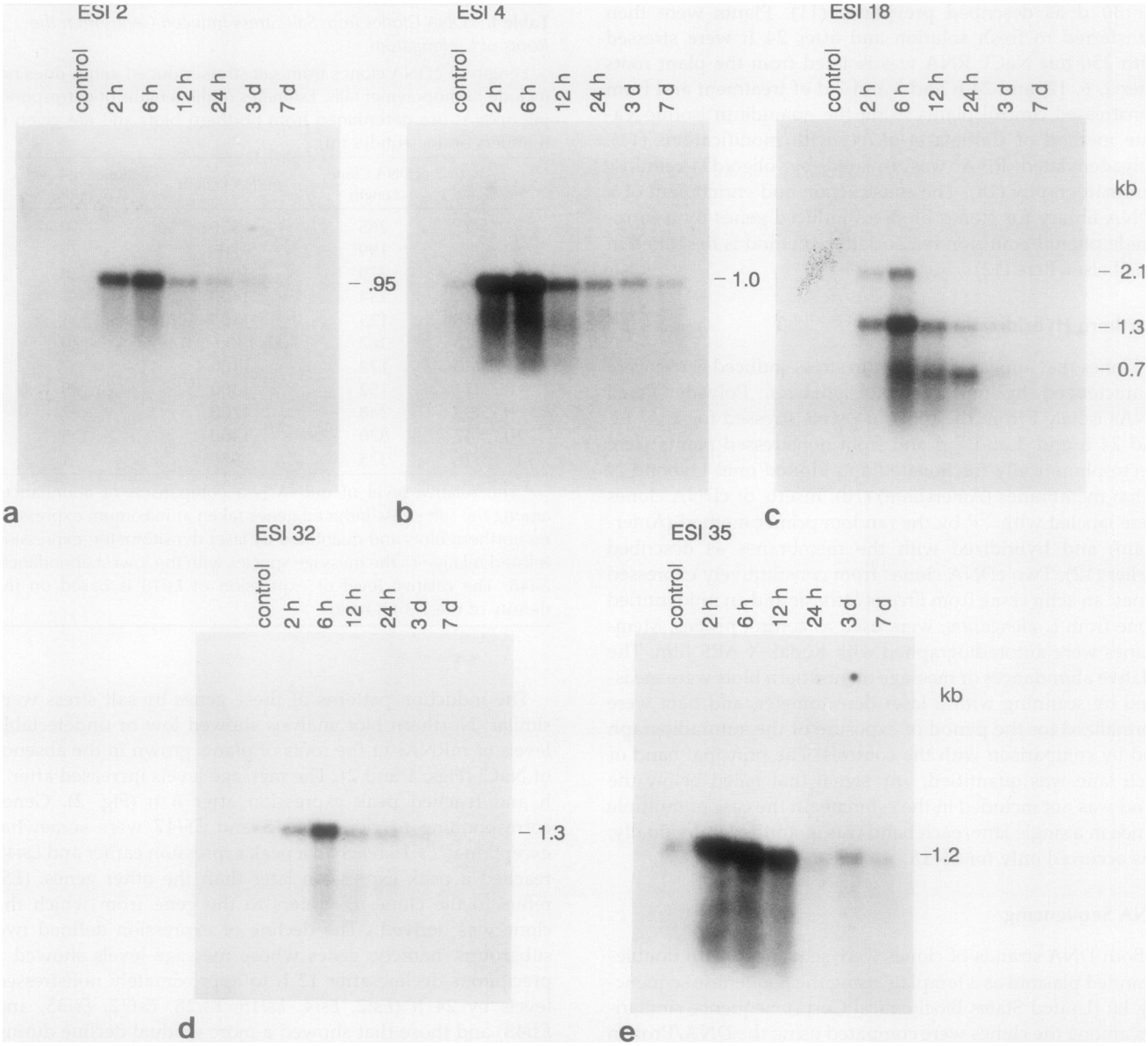

Figure 1. Northern blots of salt stress-induced genes from *L. elongatum*: *Esi*2, *Esi*4, *Esi*18, *Esi*32, *Esi*35, respectively. Lanes contain 3 μg of polyadenylated RNA from roots of plants that were either nonstressed or treated with 250 mM NaCl for 2, 6, 12, and 24 h and 3 and 7 d. Membranes were probed with inserts from cDNA clones labeled with ^{32}P nucleotides by the random primer method (Amersham). Membranes were autoradiographed at −80°C with two intensifying screens; time of exposure for the autoradiographs was (a) 4 d, (b) 4 d, (c) 4 h, (d) 14 h, and (e) 3 d. The different rate of migration of samples in the blot probed with *ESI*35 is likely an artifact of electrophoresis; two other blots probed with *ESI*35 did not show unequal migration.

similarity over its coding region with *rab*16 (formerly RAB21) (20, 24) and *dhn* genes isolated from barley and M3 from maize (6, 7). The *ESI*18 clone contains a truncated coding region for 39 amino acids; 32 of these 39 amino acids were identical with the carboxy-terminal portion of the protein coded for by the barley dehydrin clone *dhn*3 (formerly B17) (7). This region of *ESI*18 contains a 14-amino acid conserved sequence KKSLMDKIKEKLPG, which is repeated twice in the maize M3 gene, all members of barley *dhn* and rice *rab* multigene families, and two genes in *Craterostigma plantagineum* (21). A related sequence is also found once in the internal portion of the cotton LEA D11 protein (2).

DISCUSSION

Clones from 11 different genes or gene families that are either de novo induced or show enhanced expression in *L. elongatum* after the initiation of salt stress have been identified. These clones were selected originally only on the basis of higher levels of the mRNAs in roots after 6 h of salt stress

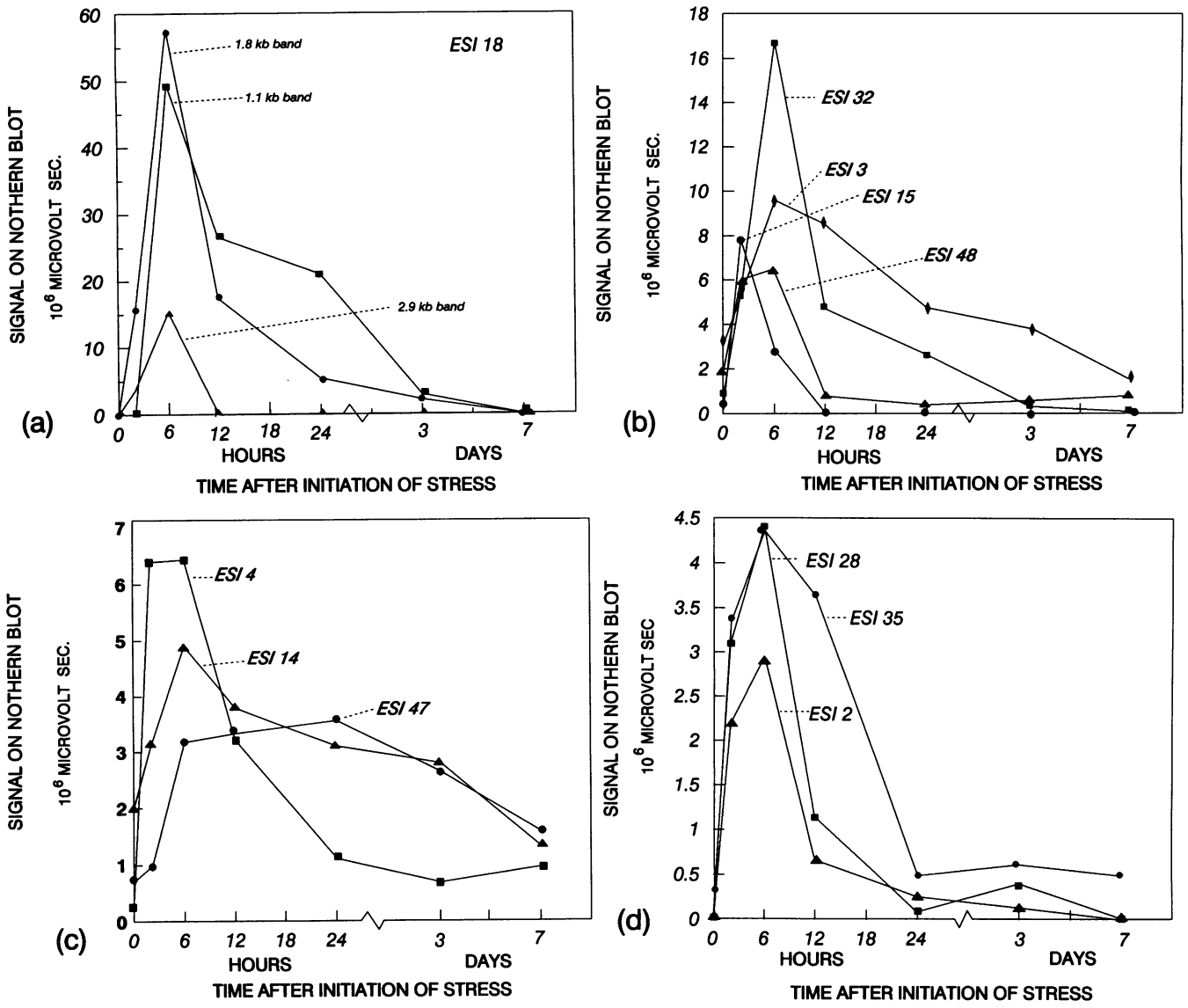

Figure 2. Relative expression of salt stress-induced genes. The relative abundance of respective mRNAs was determined by measuring the density of signal on northern blots by a laser scanning densitometer. Data were normalized for the time of exposure of the autoradiographs. Quantification was performed on a single northern blot for each clone. Comparison of the levels of maximum expression indicates that the relative abundance of mRNAs of these genes varies more than 2 orders of magnitude. Note that the *x* axis is discontinuous after 24 h.

as compared with controls. Surprisingly, all 11 genes or gene families showed similar rapid induction within 2 h of stress initiation. With the exception of *Esi*15 and *Esi*47, all reached a peak of expression by 6 h. *Esi*15 had maximum expression at 2 h, and *Esi*47 reached a peak expression later, approximately 24 h after the initiation of stress, although it had reached 90% of maximum expression by 6 h.

The expression of these 11 genes contrasts with temporal expression of a tonoplast ATPase gene that began to increase in roots of *L. elongatum* only at day 7 of stress with 250 mM NaCl (A. Galvez, P. Gulick, and J. Dvořák, unpublished results). The expression of all 11 genes returned to the basal level during salt stress; this indicates that this group of genes represents a specific and initial response. We refer to this set of genes as *early salt stress induced* and accordingly designate the genes *Esi*. Although there is a striking similarity in the pattern of the induction of *Esi* genes, the absolute level of expression among the set varied more than 2 orders of magnitude.

A sequence homology search in the GenBank data base showed significant sequence similarity to other reported sequences for only a single clone. This is not surprising considering that relatively few salt stress-inducible genes have been reported, and some of these are from quite different biological systems. Additionally, all of these clones are partial cDNAs and are likely to contain largely 3′-nontranslated sequences; additional sequence data from full-length clones would improve such comparisons.

The nucleotide sequence of *ESI*18 was found to be homologous to barley dehydrin genes *dhn*3, *dhn*9, *dhn*17, *dhn*18 (formerly B3, etc.), rice ABA-inducible genes *rab*16a, -b, -c, and -d, and the cotton gene for the LEA protein D11. These genes have been shown to be induced by desiccation (7), by ABA treatment, by salt stress (6, 20), by cold (13), and, in the case of LEA proteins, during normal seed development (2).

Clone *ESI*18 hybridized to messages of 0.7, 1.3, and 2.1 kb, which is likely due to the presence of a multigene family in *L. elongatum* whose members produce messages of different lengths and which have slight differences in the dynamics of expression. Related genes in rice and barley have been found to make up multigene families (7, 24). Partial sequencing of additional cross-hybridizing cDNA clones from *L. elongatum* support this supposition. The apparent lengths of rice and barley mRNAs are somewhat different from those observed in the roots of *L. elongatum*. Messages from 700 to 1100 nucleotides were observed in barley, and sequence data indicated messages from 700 to 900 nucleotides in rice. Like the rice and barley genes, message levels for *ESI*18 were very high; messages corresponding to a 1.3-kb band on the northern blot were 4 to 5 times more abundant than those for *Esi*2 and *Esi*32, which were the next most abundant messages of stress-induced genes.

The 10 *ESI* clones other than *ESI*18 did not show homology to other reported gene sequences in the GenBank (version summer 91). Physiological models for salt tolerance as well as genetic inheritance of the trait indicate that salt tolerance is a multigenic trait. Stress-induced genes that have been identified to date clearly represent a small fraction of the gene response to salt stress in plants.

This is the first report of the isolation of a relatively large number of cDNA clones of salt stress-induced genes of a salt-tolerant plant species. This set of similarly regulated genes provides a significant resource for the comparative study of gene expression in response to stress, especially in the genetic stocks that have been derived from *L. elongatum* and *T. aestivum* (8). The temporal characterization of the expression of the *Esi* genes indicates that the initial gene response to salt stress is complex and transient and suggests that subsequent genetic response involves additional sets of genes.

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